Insulin secretion and ASNA-1-dependent function of the endoplasmic reticulum in \textit{C. elegans}

Ola Billing
To Lotta, Irma and Lars
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF ORIGINAL PAPERS</td>
<td>vi</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>The model organism <em>C. elegans</em></td>
<td>1</td>
</tr>
<tr>
<td>ASNA-1</td>
<td>4</td>
</tr>
<tr>
<td><em>asna-1</em>-dependent tail-anchored protein targeting to the endoplasmic</td>
<td>5</td>
</tr>
<tr>
<td>reticulum</td>
<td></td>
</tr>
<tr>
<td>Tail-anchored protein-independent functions of <em>asna-1</em></td>
<td>8</td>
</tr>
<tr>
<td>Insulin/insulin-like growth factor-like factor signalling in <em>C. elegans</em></td>
<td>9</td>
</tr>
<tr>
<td>The dauer diapause</td>
<td>10</td>
</tr>
<tr>
<td>The L1 diapause</td>
<td>12</td>
</tr>
<tr>
<td>The adult reproductive diapause</td>
<td>13</td>
</tr>
<tr>
<td>Insulin-like peptides in <em>C. elegans</em></td>
<td>13</td>
</tr>
<tr>
<td>ASNA-1 in IIS and DAF-28 secretion</td>
<td>15</td>
</tr>
<tr>
<td>Endoplasmic reticulum stress</td>
<td>15</td>
</tr>
<tr>
<td>The ER unfolded protein response</td>
<td>16</td>
</tr>
<tr>
<td>Membrane remodelling during endoplasmic reticulum stress</td>
<td>18</td>
</tr>
<tr>
<td>Mitochondrial function</td>
<td>18</td>
</tr>
<tr>
<td>Protein import across the outer mitochondrial membrane</td>
<td>19</td>
</tr>
<tr>
<td>The mitochondrial unfolded protein response in <em>C. elegans</em></td>
<td>21</td>
</tr>
<tr>
<td>Mitochondrial functions in insulin secretion</td>
<td>21</td>
</tr>
<tr>
<td>Mitochondria in longevity and dietary sensing</td>
<td>22</td>
</tr>
<tr>
<td>METHODS</td>
<td>24</td>
</tr>
<tr>
<td>Mitochondrial function assays</td>
<td>24</td>
</tr>
<tr>
<td>TA protein targeting assay</td>
<td>26</td>
</tr>
<tr>
<td>Electron microscopy</td>
<td>27</td>
</tr>
<tr>
<td>AIMS</td>
<td>29</td>
</tr>
<tr>
<td>RESULTS</td>
<td>30</td>
</tr>
<tr>
<td>Identification of new genes that modify insulin signalling</td>
<td>30</td>
</tr>
<tr>
<td><em>ykt-6</em> and <em>tomm-40</em> are positive modulators of DAF-28/insulin secretion</td>
<td>33</td>
</tr>
<tr>
<td>TOMM-40 is a ubiquitously expressed mitochondrial protein</td>
<td>36</td>
</tr>
<tr>
<td>TOMM-40 is a mitochondrial translocase required for mitochondrial function</td>
<td>37</td>
</tr>
<tr>
<td>Low levels of reactive oxygen species in mitochondria stimulate DAF-28/insulin secretion</td>
<td>39</td>
</tr>
<tr>
<td><em>asna-1</em> promotes ER-targeting of the tail-anchored protein SEC-61β in vivo</td>
<td>41</td>
</tr>
<tr>
<td><em>wrb-1</em> has partially overlapping phenotypes with <em>asna-1</em></td>
<td>44</td>
</tr>
<tr>
<td>Analysis of additional predicted components of the <em>C. elegans</em> tail-anchored protein targeting machinery</td>
<td>46</td>
</tr>
</tbody>
</table>
asna-1 and wrb-1 mutants have ER and Golgi morphology defects and accumulate cytosolic inclusion bodies

**DISCUSSION**

Identification of IIS and DAF-28 secretion regulators

Functional mitochondria promote DAF-28 secretion and IIS-dependent growth

Defective Golgi precludes DAF-28/insulin secretion

DAF-28 secretion is mechanistically similar to mammalian insulin secretion

ASNA-1 and WRB-1 promote tail-anchored protein targeting

asna-1 is likely to have wrb-1-independent functions

**FUTURE DIRECTIONS**

A screen for new insulin secretagogues

Characterization of *C. elegans* insulin-like peptides

Benefits and validation of a metazoan model for tail-anchored protein targeting

**CONCLUSIONS**

**ACKNOWLEDGEMENTS**

**REFERENCES**

**ORIGINAL PAPERS**
ABSTRACT

ASNA\textsubscript{1} is a well-conserved ATPase involved in a wide range of functions, including cisplatin resistance, growth control, insulin secretion and targeting of tail-anchored (TA) proteins to membranes. It is a positive regulator of insulin secretion both in the roundworm \textit{Caenorhabditis elegans} and in humans. Insulin secretion and downstream insulin/IGF signalling (IIS) stands at the heart of many human pathologies, such as diabetes, Alzheimer’s disease and cancer. A better understanding of IIS may therefore prove vital for treatment and cure of these diseases. This thesis aims to further investigate the function of \textit{asna-1}, and to identify new regulators of IIS based on the \textit{asna-1} phenotype in \textit{C. elegans}.

Worms lacking ASNA-1 arrest growth in the first larval stage, L\textsubscript{1}, with reduced insulin secretion. The L\textsubscript{1} arrest represents the strongest of the IIS phenotypes in worms. Most regulators of the insulin pathway have been identified in screens for other IIS phenotypes, influencing lifespan or the dauer diapause. Therefore, new regulators could be found by screening for genes which, when inactivated, cause an \textit{asna-1}-like L\textsubscript{1} arrest. Using bioinformatic approaches, a set of 143 putative \textit{asna-1} interactors were identified, based on their predicted or confirmed interaction with \textit{asna-1} in various organisms. Depletion of the Golgi SNARE homologue YKT-6 or the mitochondrial translocase homologue TOMM-40 caused \textit{asna-1}-like larval arrests. Using several criteria, including genetic suppression by \textit{daf-16}/Foxo, it was established that YKT-6 and TOMM-40 are positive regulators of IIS. Both proteins were also required for normal DAF-28/insulin secretion.

Further investigation of TOMM-40 identified it as a ubiquitously expressed mitochondrial translocase in \textit{C. elegans}: It localized to mitochondrial membranes and was required for importing a tagged mitochondrial reporter across mitochondrial membranes. Depletion of TOMM-40 caused a collapse of the proton gradient across the inner mitochondrial membrane and triggered the mitochondrial unfolded protein response (UPR). Worms with defective mitochondria failed to grow normally in presence of food, but this growth defect was suppressed by \textit{daf-16(mgDf50)}. In addition, \textit{tomm-40(RNAi)} led to DAF-16/FOXO activation, an effect that was suppressed by over expression of DAF-28/insulin. Taken together, these findings support a model whereby signals of food availability are conveyed through respiring mitochondria to promote DAF-28/insulin secretion, which in turn promotes growth.
Biochemical studies have identified ASNA-1 as a chaperone that targets a subset of newly synthesized TA proteins to a receptor at the endoplasmic reticulum (ER) membrane. However, these findings have not been tested in vivo in a metazoan model. A reporter-based system to analyse TA protein targeting into the ER in live animals using confocal microscopy was set up. A model asna-1-dependent TA protein, Y38F2AR.9/SEC-61β, required functional ASNA-1 for correct targeting to the ER. Conversely, a model asna-1-independent TA protein, CYTB5.1/cytochrome B5, did not. This phenotype was shared with the predicted asna-1 receptor homologue, wrb-1. Consistently, WRB-1 was found to localize to the ER. However, other wrb-1 mutant phenotypes only partially overlap with those of asna-1 mutants, suggesting that ASNA-1 is either partially independent of WRB-1 for TA protein targeting or that ASNA-1 has additional functions besides its role in TA protein targeting.

Confocal microscopy also indicated that the ER morphology was aberrant in asna-1 and wrb-1 mutants. ER UPR was elevated in the asna-1 mutants, as indicated by the upregulation of an hsp-4/BiP reporter. Transmission and immuno-electron microscopy of these mutants revealed a swollen ER lumen, which is another hallmark of ER stress. High levels of autophagy in asna-1 animals and the presence of ER-containing autophagosomes in both asna-1 and wrb-1 mutants indicated a stress-induced remodelling of the ER membrane in these two mutants. In addition, both mutants had normal mitochondrial morphology, but showed severe effects on Golgi compartment morphology. Hypothetically, all these phenotypes could be due to defects in the signal recognition particle (SRP) pathway. This is because Y38F2AR.9/SEC-61β is both a TA protein and a component of the SEC-61 translocon. However, both Golgi and ER morphology was normal in Y38F2AR.9/sec-61β(tm1986) mutant animals, suggesting that the organellar defects seen in asna-1 and wrb-1 were due to a TA protein-dependent mechanism rather than an SRP-dependent mechanism. In addition, asna-1 mutants displayed numerous protein aggregates, consistent with a proposed role for ASNA-1 in shielding aggregation-prone TA protein membrane anchors from the hydrophilic environment of the cytosol.

In conclusion, YKT-6 and TOMM-40 are positive regulators of IIS and DAF-28/insulin secretion, implicating roles for Golgi and mitochondria in IIS. DAF-28 is a metabolically regulated insulin in C. elegans, since its secretion depends on active mitochondria. Mutants for asna-1 and its predicted receptor wrb-1 show severe defects in ER and Golgi morphology. These defects may occur because TA protein targeting in asna-1 and wrb-1 mutants is defective, which is also demonstrated here in the first analysis of this process in live animals.
ABBREVIATIONS

ADP: Adenosine diphosphate
ArsA: Arsenite stimulated ATPase
ASNA-1: Arsenite ATPase transporter 1
ATP: Adenosine triphosphate
DA: Dafachronic acid
DNA: Deoxyribonucleic acid
dsRNA: Double-stranded ribonucleic acid
EM: Electron microscopy
ER: Endoplasmic reticulum
ERA: Endoplasmic reticulum-containing autophagosome
GDP: Guanosine diphosphate
GTP: Guanosine triphosphate
IIS: Insulin and insulin-like growth factor signalling
Immuno-EM: Immuno-electron microscopy
IMS: Inter membrane space
mtUPR: Mitochondrial unfolded protein response
RER: Rough endoplasmic reticulum
RNA: Ribonucleic acid
RNAi: RNA interference
SNARE: Soluble NSF attachment protein receptor
SRP: Signal recognition particle
TA: Tail-anchored
TEM: Transmission electron microscopy
TMD: Transmembrane domain
TMRE: Tetramethylrhodamine ethyl ester
UPR: Unfolded protein response
∆Ψ: Mitochondrial membrane potential
LIST OF ORIGINAL PAPERS

Paper I


Paper II


Paper III

INTRODUCTION

Animals need to be able to cope with a potentially stressful environment, where temperature, chemical composition and food availability can rapidly change. During the course of evolution, animals have acquired means to cope with these various types of stresses, such that living conditions are sensed and conveyed into appropriate cellular, behavioural and developmental responses. The cell signalling networks underlying appropriate adaptations to changes in the environment are evolutionary ancient and failure in these networks are linked to many severe human diseases, demonstrating the evolutionary pressure of their existence.

Research in our lab has shown that the phylogenetically well-conserved gene asna-1 plays an important role in coupling food availability to growth and insulin secretion in the roundworm Caenorhabditis elegans and in humans (Kao et al., 2007). Our lab also showed that asna-1 mediates resistance to the chemotherapeutic drug cisplatin (Hemmingsson et al., 2010). Other studies, mostly performed in vitro have shown that ASNA-1 also acts as a chaperone and aid in targeting of a special class of membrane proteins, the tail-anchored (TA) proteins, to the endoplasmic reticulum (ER) (Mateja et al., 2009; Schuldiner et al., 2008; Stefanovic and Hegde, 2007). It is currently unclear if the roles of ASNA-1 in insulin secretion and TA protein targeting are interdependent or if they represent two independent functions.

This thesis employs functional studies of asna-1 in C. elegans. The first part aims to identify new regulators of insulin/IGF signalling (IIS) based on the asna-1 phenotype in C. elegans. Through a detailed characterization of one of these new regulators, mitochondria-dependent insulin secretion is investigated. The second part of the thesis aims to model TA protein targeting to the ER in live C. elegans animals. Through the studies of TA protein targeting in the context of a live multicellular animal, we find evidence suggesting independent functions of asna-1 in TA protein targeting and growth control. Given the clinical bearing of asna-1 on diabetes and cancer, a better understanding of asna-1 functions may help to develop new strategies to improve treatment of these diseases.

The model organism C. elegans

The ecology of C. elegans in nature is poorly understood. Laboratory strains have mostly been isolated from nutrient-rich environments such as rotten fruits and other decaying organic matter. In the laboratory, worm populations are sustained on agar plates, supplemented with salts and
seeded with strains of *E. coli* bacteria. Maintenance in lab environments is therefore cheap and easy. As an experimental model system, *C. elegans* has many other advantages. With a generation rate of approximately 3.5 to 4 days at 20°C and an average lifespan of 3 to 4 weeks, obtaining experimental data in a large number of animals is a fast process. Studies *in vivo* are simplified by the fact that fully-grown adults are about 1 mm long and transparent. Consequently, physiological processes such as feeding and defecation can easily be studied using dissection microscopes. In addition, the expression of labelled gene reporters can be visualized in sedated live animals under high power microscopy. This enables in-depth analysis of the localization and movement of tagged proteins and other markers in the context of a whole live organism. In 1998, the complete genomic sequence of *C. elegans* was published (Consortium, 1998). This has revealed that around 60% of human genes have a homolog in *C. elegans*, implying that functional studies of genes in *C. elegans* are relevant to human biology. The complete cell lineage of *C. elegans* has been determined and the complete wiring diagram of its nervous system has been outlined. In addition, there is a wide array of other powerful genetic tools to investigate gene function in *C. elegans*. Some of these are discussed below.

In *forward genetics*, mutagenic agents are used to introduce mutations in the genome. Isolated mutants with interesting phenotypes are then mapped or sequenced to reveal sites of mutations. Forward genetics is particularly efficient in suppressor screens to find interactors. If the gene under study has a detectable mutant phenotype, changes in a given interacting gene may suppress or enhance that phenotype. Successfully employed, suppressor/enhancer screens can identify interacting genes and even pinpoint relevant protein domains that are of particular importance for its interaction. Importantly, whole genome sequencing analysis has dramatically speeded up the process of identifying the genes that have been mutated in forward mutagenesis.

*Reverse genetics* on the other hand starts with a gene sequence of interest. Functional studies are then performed on that particular sequence through gene knockout or gene silencing. A fast way of achieving targeted gene silencing in *C. elegans* is to administrate double stranded RNA (dsRNA) complementary to the gene under study. This will induce the RNAi machinery, which is part of an innate immune response to foreign viral RNA plasmids (Wilkins et al., 2005). Injecting or soaking animals in dsRNA solutions, or feeding them with plasmids expressing dsRNA under control of *C. elegans* promoters can be used to administrate the dsRNA (Fire et al., 1998; Tabara et al., 1998; Timmons et al., 2001). The latter also allows for tissue-directed silencing through tissue-specific promoters in mutant
backgrounds that inhibit systemic spreading of RNAi (Qadota et al., 2007). The use of RNAi to rapidly achieve gene silencing was greatly boosted by the complete sequencing of the C. elegans genome (Consortium, 1998). However, RNAi techniques also have some drawbacks. Some genes are refractory to RNAi knockdown and for others RNAi can fail to completely deplete gene function. The effects can vary between experiments, which together with potential non-specific targeting calls for caution when employing RNAi. In addition, nerve cells are refractory to dsRNA uptake, since they lack receptors for dsRNA uptake (Shih and Hunter, 2011). However, this can be circumvented by performing RNAi in a genetic background that allows neurons to take up dsRNA (Calixto et al., 2010) or by delivering dsRNA with neuronally expressed transgenes.

Another way to target specific genes is by generating deletion alleles in the gene of interest. Many laboratories have generated libraries of strains carrying such alleles and strains carrying mutations in specific genes have been isolated. By collecting, generating and distributing such mutant strains, organizations like the C. elegans knockout consortium and the National Bioresearch Project have contributed greatly to the field. In addition, mutations can be introduced by generating site-directed, double stranded DNA (dsDNA) breaks. Such DNA breaks are repaired in a process called non-homologous end joining, which often generate indels. Instead of allowing end joining, homologous recombination can be used over the double stranded DNA breaks, to replace wild-type gene sequence with cloned sequences (Gloor et al., 1991; Orr-Weaver et al., 1981). This method enables researchers to tag genes in their endogenous context and to create point mutations. While this technique has been known for some time, its use in C. elegans has previously been constrained since it has relied on endogenous transposons, parasitic DNA elements able to move in their host genome and create double stranded DNA breaks as they are excised. The use of endogenous transposons has the big disadvantage that they exist in many copies, creating a heavy mutational load as they are excised. But with the creation of a library of strains with single Mos1 transposable elements from Drosophila mauritiana in C. elegans, the use of transposon techniques was greatly improved (Bazopoulou and Tavernarakis, 2009; Robert and Bessereau, 2007). However useful in targeted deletion and insertion of single copy transgenes (Frokjaer-Jensen et al., 2008), a drawback of this technique is its dependence on an available Mos1 element in the vicinity of the gene under study.

Through recent scientific proceedings, site-directed mutagenesis can now also be obtained using the Cas9/CRISPR endonuclease system, which does not depend on transposons. Here, mediators of an adaptive bacterial
immune response have been adapted for the purpose of inducing double stranded DNA breaks in the C. elegans genome (Chen et al., 2013a; Friedland et al., 2013). Because the sequences required for Cas9 targeting are very common in the C. elegans genome and because the techniques required are simple, the Cas9/CRISPR system is likely to speed up gene analysis greatly in an already tractable model system.

**ASNA-1**

ASNA1 was first identified as the human homolog of the bacterial ArsA protein (Kurdi-Haidar et al., 1996). In bacteria, ArsA acts as the catalytic subunit of a metal efflux pump that provides resistance to arsenic and antimonite (Rosen et al., 1999). Cross-resistance to arsenic and antimonite was found in several human cancers that were also resistant to the platinum-based, chemotherapeutic drug cisplatin. Since resistance is a main obstacle in treatment with platinum-based drugs, a clinical incentive has driven the investigation of its underlying mechanisms. Work from our group has shown that ASNA1 is upregulated in cisplatin-resistant cell lines and that downregulation of ASNA1 causes hypersensitivity to cisplatin in both cell lines and in C. elegans (Hemmingsson et al., 2010; Hemmingsson et al., 2009a; Hemmingsson et al., 2009b). These studies have identified ASNA1 as a potential target to circumvent treatment failure due to platinum-based drug resistance.

While bacterial ArsA is involved exclusively in metal resistance, eukaryotic versions of the gene have acquired additional functions. The yeast homologue Get3 is involved in the secretory pathway and is required for secretion (Schuldiner et al., 2005). C. elegans and human homologs of ASNA-1 are both required for insulin secretion1 (Kao et al., 2007). The underlying causes for secretory defects in ASNA-1-depleted worms or in human cell models are still unknown, but in yeast they might be caused by defective Golgi function (Schuldiner et al., 2005). The yeast homolog Get3 (an acronym for Golgi-to-Endoplasmic reticulum Transport) was identified in an effort to find genes that were involved in the early secretory pathway. Get3 mutants were found to be defective in retrograde Golgi-ER transport, since a reporter with the endoplasmic reticulum (ER) retention sequence HSEL was secreted instead of transported back to the ER. More recently, the GET acronym has acquired another meaning as Guided Entry of Tail-anchored proteins (Schuldiner et al., 2008).

---

1 The role of ASNA-1 in IIS is discussed further in the section "ASNA-1 in IIS and DAF-28 secretion".
**asna-1-dependent tail-anchored protein targeting to the endoplasmic reticulum**

Nucleus-encoded membrane proteins are synthesized on cytosolic ribosomes and can be targeted to the ER, to mitochondria or to peroxisomes. Insertion into the ER allows subsequent targeting to the nuclear membranes, to the plasma membrane or to membranes in secretory and endocytic vesicles. In the process of targeting to membranes, the hydrophobic membrane anchors of these proteins need to be protected from exposure to the hydrophilic environment of the cytosol. A failure to do so can cause folding and aggregation problems (Powis et al., 2013; Rampelt et al., 2012). In co-translational targeting to the ER, this problem is solved by a direct association between the ribosome and the SEC61 protein translocon at the ER membrane. As a nascent chain containing an N-terminal signal sequence emerges from a translating ribosome, it is recognised by the signal recognition particle (SRP), which mediates association with the SEC61 translocon through a receptor (**Figure 1A**). The membrane protein is then directly inserted into the translocon pore at the ER membrane as translation proceeds [Reviewed in (Nyathi et al., 2013)].

Tail-anchored (TA) proteins, on the other hand, represent a separate class of membrane proteins with 325 members in humans (Kalbfleisch et al., 2007), including members of the SNARE family, the Bcl2-family and subunits of the mitochondrial TOM-complex. Two structural characteristics of these proteins make them utilize a post-translational route for membrane targeting. First, they have a single transmembrane domain (TMD) located very close to the C-terminus. Second, their N-termini lack a signal sequence and thus they cannot be recognized by the SRP. Instead, recent biochemical and structural studies using yeast or mammal components, has outlined a separate pathway for targeting of TA proteins to the ER membrane (Mateja et al., 2009; Schuldiner et al., 2008; Stefanovic and Hegde, 2007). The principal mechanisms in this pathway are similar in yeast and in humans (**Figure 1B**): When a nascent TA protein has been translated by the ribosome, a ribosome-associated pre-targeting complex binds to its hydrophobic TMD (Fleischer et al., 2006; Mariappan et al., 2010). The pre-targeting complex hands over the TMD to a hydrophobic groove presented by a symmetric and ATP-bound homodimer of the ASNA1 protein (Mateja et al., 2009; Suloway et al., 2009). The ASNA1-TA protein complex then moves to the ER membrane and associates with a heterodimer receptor, composed of two subunits. The first subunit, Get2 in yeast and CAML in mammals, tethers ASNA1 to the ER membrane. The second subunit, Get1/WRB, mediates substrate release (Mariappan et al., 2011; Vilardi et al., 2011; Wang et al., 2011; Yamamoto and Sakisaka, 2012). The ATP hydrolysis of ASNA1
precedes substrate release at the ER membrane, but may even be required in an earlier step to weaken the interaction with the pre-targeting complex after TMD binding (Hegde and Keenan, 2011). At the ER membrane, the Get1/WRB receptor subunit interacts with ADP-bound ASNA-TMD and inserts its cytosolic residue into the interface of the ASNA1 dimer, which causes it to open up and release the TMD and ADP.

Figure 1. Membrane protein targeting to the endoplasmic reticulum in *S. cerevisiae*

A. In the co-translational targeting pathway, a hydrophobic signal peptide is captured by the SRP as it emerges from the ribosome. The SRP-ribosome complex associates with an SRP receptor in a GTP-dependent manner at the ER membrane. Upon docking to the Sec61 translocon, insertion of the membrane protein into the ER proceeds co-translationally.

B. In the post-translational targeting pathway, a hydrophobic transmembrane domain (TMD) is captured by a pre-targeting complex. The TMD is then loaded onto an ATP-bound Get3 dimer, which targets to a receptor at the ER membrane. ATP hydrolysis, release of ADP and stabilization of the open configuration of the Get3 dimer by Get1 drive release of the TA protein at the ER membrane. The figure is reprinted by permission from Macmillan Publishers Ltd: Nature (Hegde and Keenan, 2011), copyright (2011).
Binding of new ATP molecules to the ASNA1 dimer renews its closed configuration and causes it to disassociate from the Get1/WRB receptor (Mariappan et al., 2011; Stefer et al., 2011). ASNA1 is then able to associate with the pre-targeting complex and engage in a new cycle of TMD targeting (Mateja et al., 2009). Structural and biochemical analysis revealed that the dimerization of the ASNA1 protein and its ATPase function is required for its interactions with TA proteins, the pre-targeting complex and receptors at the ER membrane (Chartron et al., 2010; Wang et al., 2011). Formation of the homodimer in Get3 depends on a pair of conserved cysteins at the interface between the two proteins, cys285 and cys288 (Mateja et al., 2009).

While ASNA-1 and some other proteins involved in this pathway are conserved from yeast to humans, others are not (Table 1). The pre-targeting complex members Get4/TRC35/CEE-1 and Sgt2/SGTA/SGT-1 are conserved in yeast, mammals and C. elegans, but the Get5/UBL4A member has no obvious C. elegans homologue. BAG6 is only present in mammals. The ER receptor also seems to have taken different forms in different species.

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Mammals</th>
<th>C. elegans</th>
</tr>
</thead>
<tbody>
<tr>
<td>---</td>
<td>CAML</td>
<td>---</td>
</tr>
<tr>
<td>Get1</td>
<td>WRB</td>
<td>WRB-1</td>
</tr>
<tr>
<td>Get2</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Get3</td>
<td>ASNA1</td>
<td>ASNA-1</td>
</tr>
<tr>
<td>Get4</td>
<td>TRC35</td>
<td>CEE-1</td>
</tr>
<tr>
<td>Get5</td>
<td>UBL4A</td>
<td>---</td>
</tr>
<tr>
<td>Sgt2</td>
<td>SGTA</td>
<td>SGT-1</td>
</tr>
<tr>
<td>---</td>
<td>BAG6</td>
<td>---</td>
</tr>
</tbody>
</table>

*Table 1. Identified homologs of proteins involved in post-translational targeting of tail-anchored proteins to the ER*

Light grey indicates receptor homologs and dark grey indicate members of the cytosolic pre-targeting complex. Dashed lines indicate that no obvious homologue has yet been identified.

Get2 is only found in yeast and the CAML protein is mammal-specific. Although structurally unrelated, the function of Get2 and CAML is similar in that they both recruit the ASNA-1-TA protein complex to the ER membrane. For the second component of the receptor, sequence homology and structure similarity is shared between yeast Get1 and mammalian WRB. Both have a three transmembrane domain topology and a conserved coiled-coil domain that is exposed to the cytosol. The *C. elegans* WRB-1 protein has a weakly predicted cytosolic coiled-coil domain [prediction software: (Lupas et al., 1991)] and share both primary sequence homology and a three transmembrane domain topology [prediction software: (Kall et al., 2004)] with WRB. Importantly, the coiled-coil domain in Get1 and WRB was
identified as the binding site for Get3 and ASNA1 respectively (Stefer et al., 2011; Vilardi et al., 2011). In Get1, this domain acts to stabilize a substrate-free configuration the Get3 homodimer, thereby promoting its TA substrate release (Stefer et al., 2011). It was also demonstrated that Get1 is completely indispensible for Get3-mediated TA protein targeting to the ER.

A model TA protein shown to depend on ASNA1 for targeting to ER membranes in vitro is SEC61β (Favaloro et al., 2008; Stefanovic and Hegde, 2007). This protein is part of the Sec61α-β-γ heterotrimer, which forms the minimal component of the SEC61 translocon pore. In this way, SEC61β could be required for SRP-dependent, co-translational insertion of nascent non-TA proteins (Knight and High, 1998; Simon and Blobel, 1991). But other evidence suggests a subsidiary role for SEC61β in co-translational protein integration into the ER (Kelkar and Dobberstein, 2009).

Some other TA proteins are targeted to the ER even in the absence of ASNA1. Cytochrome B5 is a TA protein that is inserted into ER membranes independently of ASNA1. ER targeting of cytochrome B5 is also independent of the SEC61 translocon, suggesting that it targets to membranes via an unassisted insertion mechanism (Cross et al., 2009). Instead, the charged C-terminal sequence of cytochrome B5 along with the specific lipid composition of the ER membrane are thought to give specificity to its ER localization (Brambillasca et al., 2005; Henderson et al., 2007).

**Tail-anchored protein-independent functions of asna-1**

Although shown to be important for targeting of many TA proteins to the ER membrane, ASNA-1 homologs seem to have additional functions that are independent of TA protein handling. When copper is available in the cytosol, yeast Get3 is able to bind the C-terminal end of the chloride transporter Gef1, which is not a TA protein (Metz et al., 2006). In search for metal binding motifs that could promote such an interaction, it was shown that cys285 and cys288, the same pair of cysteins required for Get3 dimerization and TA protein binding, were required for the ability of Get3 to bind Gef1. Mutation of these two cysteins inhibited growth during metal stress. In a second study, it was demonstrated that Get3 acts as a guanine exchange factor (GEF). Get3 was shown to bind the G-protein Gα subunit Gpa1 and promote its exchange of GDP for GTP (Lee and Dohlman, 2008). This binding of Get3 to Gpa1 was enhanced by copper exposure. Subsequent to the copper exposure, activation of mitogen-activated protein kinase (MAPK) suggested a Get3-MAPK signalling axis during metal stress. But also here, dimerization of Get3 was required for pathway activation. These two studies revealed that a metal response mechanism involving Get3 exists in yeast, but
they also indicate that this mechanism might not be completely separable from TA protein functions since both functions seem to rely on Get3 dimerization. In addition, mutations in either Get1 or Get2 cause sporulation defects (Auld et al., 2006). By contrast, a mutation in Get3 does not recapitulate the sporulation defects of Get1 and Get2, but rather suppresses them. Also, the transcription of Get3 is co-regulated with Npl4, which is a component in the ER associated decay (ERAD) machinery. A mutation in Get3 can suppress many of the Npl4 mutant phenotypes. But Get1 and Get2 are neither co-regulated with Npl4, nor can they suppress Npl4 mutant phenotypes. These lines of evidence clearly indicate separable roles for Get3 and Get1/Get2 in yeast cell biology. This in turn indicates that all the pleiotropic phenotypes of Get3 mutants are not likely to solely depend on its interactions with the Get1/Get2 receptor.

Other indications of TA protein-independent roles of ASNA-1 came from studies in C. elegans. Expression of a cys285ser, cys288ser double mutant version of asna-1 in asna-1 mutants, could not rescue the cisplatin sensitivity phenotype. However it did rescue IIS-mediated growth defects, indicating that asna-1 may promote IIS through a mechanism that is independent of TA proteins (Hemmingsson et al., 2010). Another study in C. elegans also shows that ASNA-1 binds to several non-TA proteins that are seemingly unrelated to the TA-protein targeting machinery (Natarajan, 2012).

An in vitro study using mammal components showed that ASNA1 could also bind to short secretory proteins, even though they possessed signal sequences. These short secretory proteins utilized ASNA1 for posttranslational targeting to the ER in a pathway that also involved WRB and the Sec61 translocon (Johnson et al., 2012). Therefore, secretion defects in mammalian systems depleted of ASNA1 may be caused by a direct failure to translocate secretory proteins across the ER membrane.

**Insulin/insulin-like growth factor-like factor signalling in C. elegans**

The evolution of insulin/insulin-like growth factor -like signalling (IIS) predates the appearance of vertebrates, several hundred million years ago. The outline of the IIS pathway in C. elegans and in other organisms has confirmed a strong phylogenetic conservation of the pathway (McElwee et al., 2007). IIS in worms governs several aspects of life history in relation to food availability and stress conditions (Reviewed in (Murphy and Hu, 2013)): Under favourable conditions, worms go through embryonic development, four larval stages, L1 to L4, and an adult stage (Figure 2). But under unfavourable conditions, such as high population density, high
temperature or starvation, they can instead enter hibernating life stages at specific developmental time points. These are 1) a reversible developmental quiescence at the L1 stage and 2) a diapause in the dauer stage and 3) a reproductive diapause at the adult stage.

**Figure 2. The lifecycle of C. elegans hermaphrodites**

During reproductive growth, newly hatched embryos go through four larval stages (L1-L4) to reach adulthood. But under adverse living conditions they can arrest growth in the L1 stage or in the dauer stage. Upon return to improved living conditions, arrested L1 and dauer larvae re-enter the reproductive life cycle. Adapted from (WormAtlas, 2002-2012).

**The dauer diapause**

Worms can sense their environment through ciliated neurons that project to the outside environment in the aphids, two invaginations of the cuticle on either side of the mouth. Ciliated neurons, such as ASI and ASJ, sense environmental conditions and govern the dauer arrest accordingly (Bargmann and Horvitz, 1991): Entry into the dauer stage is promoted when temperature is high, when food is limiting and when population density is high. Under such unfavourable conditions, a late L1 larva can undergo a number of behavioural and morphological alterations (Riddle and Albert, 1997). First, it feeds and stores fat. At the L2/D stage, it stops feeding, undergoes radial constriction and seals its pharynx, completely relying on its stored energy reserves. Third, it secretes a thick cuticle with threads called alae for rapid movement.

Population density is sensed by the presence of pheromones called dauer pheromones, which are continuously secreted by *C. elegans* and concentrate with increased population density (Golden and Riddle, 1984). In the cilia of the amphid neurons, G-protein-coupled receptors (GPCRs) are known to sense food odorants (Troemel et al., 1997; Wes and Bargmann, 2001) and dauer pheromones (Kim et al., 2009) in the surrounding environment. GPCRs typically transmit signals via G proteins, and several mutants in G proteins cannot sense dauer pheromone properly (Lans and Jansen, 2007; Zwaal et al., 1997). In addition, the guanylyl cyclase DAF-11 promotes non-
dauer development by generating cGMP (Birnby et al., 2000; Vowels and Thomas, 1994). High levels of cGMP act on a cGMP-gated channel, encoded by \textit{tax}-2 and \textit{tax}-4, causing ion influx and membrane depolarization. Membrane depolarization then triggers dense-core vesicle (DCV) fusion to the plasma membrane and secretion of insulins, like DAF-28, and DAF-7/TGFβ².

Figure 3. Insulin and TGFβ signalling in \textit{C. elegans}

Hallmarks of food and population density are sensed by receptors present in the cilia of amphid neurons, such as ASI neurons. When food levels are high and population density is low, ASI neurons secrete insulin-like peptides (ILPs), such as DAF-28, and the TGFβ homolog DAF-7. Binding of ILPs and TGFβ to cell-surface receptors presented by endocrine cells, triggers insulin/IGF signalling (IIS) and TGFβ signalling cascades respectively. Activated IIS and TGFβ signalling synergistically promote synthesis of the steroid hormone dafachronic acid (DA). In target tissues, binding of DA to the nuclear hormone receptor DAF-12 promotes reproductive growth and inhibits dauer formation. Adapted from (Von Stetina et al., 2007).

² DCV secretion is reviewed in more detail in the section “Insulin-like peptides in \textit{C. elegans}” below.
Downstream of these events, the decision to enter the dauer stage is further regulated by signalling through the TGFβ pathway and the IIS pathway in endocrine cells (Figure 3) (Ohkura et al., 2003; Schaedel et al., 2012). But under adverse conditions, when IIS signalling is low, DAF-16 is not phosphorylated and is able to enter nuclei to activate transcription of genes involved in dauer development, metabolism, cellular stress response and longevity (Lee et al., 2003; McElwee et al., 2003; Murphy et al., 2003). It should be noted also that several other pathways converge on DAF-16, which can be activated by other proteins such as JNK-1 (Oh et al., 2005) in the IRE-1 branch of the endoplasmic reticulum unfolded protein response. However, in parallel to the IIS pathway, signalling through the TGFβ pathway promotes non-dauer development through secretion of a TGFβ ligand, DAF-7. In peripheral tissues DAF-7 binds to its receptor that consist of DAF-1/type1 receptor and DAF-4/type2 receptor. The receptor then phosphorylates and activates DAF-8 and DAF-14 SMADs. These components translocate to the nucleus and inhibit the function of DAF-3 SMAD and DAF-5 Sno/Ski. But when TGFβ signalling is low, DAF-3 and DAF-5 are disinhibited and promote dauer development.

Under favourable conditions, combined signalling through IIS and TGFβ pathways acts synergistically in the endocrine XXX cells in the head to promote expression of daf-9, which is involved in the synthesis of the steroid hormone dafacronic acid (DA) (Fielenbach and Antebi, 2008). When DA secretion from XXX cells reaches a threshold, a positive feedback loop from the epidermis locks development to a reproductive lifecycle (Schaedel et al., 2012). Sustained levels of DA then promote non-dauer development in target tissues by binding to the nuclear hormone receptor DAF-12, which in its DA-bound form promotes reproductive programs and inhibits dauer programs.

The L1 diapause

While TGFβ signalling is important in regulating the dauer diapause, it is not involved in the regulation of the earlier L1 arrest. Instead, IIS has been found to have a more prominent role in this diapause. Worms that hatch in the absence of food arrest growth reversibly in the L1 stage. This is also seen in a strong loss-of-function allele of daf-2, even though these animals are able to feed. Also, since daf-16/Foxo is epistatic to daf-2 in regulating the L1 arrest, the IIS pathway appears to signal through the same cascade in regulation of the L1 arrest (Baugh and Sternberg, 2006) as in regulation of the dauer

---

3 Reviewed separately in the “endoplasmic reticulum stress” section.
arrest (Hu, 2007). Similar to daf-16 mutants, a mutant in the microRNA miR-235 is also L1 arrest defective (Kasuga et al., 2013). During the L1 arrest, miR-235 is upregulated by DAF-16 in the hypodermis and glial-like cells surrounding chemosensory neurons. There it acts to arrest postembryonic development by promoting the expression of the mammalian germ cell nuclear factor ortholog nhr-91/GCNF, which in turn promotes the L1 arrest program.

**The adult reproductive diapause**

In adult hermaphrodites, starvation can induce a reproductive diapause, in which animals form no more than two embryos that are retained in the uterus (Angelo and Van Gilst, 2009). In arrested adults, the entire germline, except for a small pool of germline stem cells, undergo apoptotic cell death and the animals extend their lifespans. When these animals are put back on food, their entire germline regenerates. Although germline shrinkage was initially proposed to provide energy for the survival of starved mothers, another study suggested that it instead provided energy to developing oocytes (Seidel and Kimble, 2011). The survival of starving hermaphrodites was in this latter study instead shown to correlate with the failure of producing viable offspring during starvation.

**Insulin-like peptides in C. elegans**

Although there appears to be only one insulin receptor in C. elegans, there are 40 predicted insulin-like peptides (ILPs) (Husson et al., 2007; Li et al., 2003; Pierce et al., 2001). These contain the hallmarks of insulin peptides with an A chain peptide, a B chain peptide and a signal sequence. In addition, they are all predicted to form at least three disulphide bonds between conserved cysteine residues (Pierce et al., 2001). A subset of these ILPs also forms the canonical tertiary structure of mammalian insulin and one of these insulins, INS-6, can bind and activate the human insulin receptor (Hua et al., 2003).

The regulated temporal and spatial secretion of different ILPs governs decisions whether or not to enter the dauer stage and whether or not to leave the dauer stage (Cornils et al., 2011). INS-1 is a DAF-2 antagonist that act to both promote dauer entry and to inhibit dauer exit. On the other hand, DAF-28 and DAF-6 both have growth-promoting activities, where DAF-28 is more important in preventing dauer entry and DAF-6 is more important in promoting dauer exit. Moreover, DAF-6 expression is spatially switched from growth-promoting ASI neurons to dauer-exit-promoting ASJ neurons during the dauer arrest. There it acts to promote exit from the dauer stage.
upon improved living conditions. However, it was noted that although expression of INS-6 was up in ASJ during the dauer stage, levels were not further increased upon signals of improved living conditions. This suggests that secretion is another level of control utilized to gate the effect of this insulin.

Other insulins, like *ins-3* and *ins-33* are known to affect germline proliferation (Michaelson et al., 2010) and *ins-7* and *ins-18* affect lifespan (Murphy et al., 2003). In addition, *ins-1* and *ins-7* are involved in salt (Tomioka et al., 2006) and olfactory (Chen et al., 2013b) learning respectively.

Secretion of ILPs in *C. elegans* appears similar to secretion of human insulin in its dependence on the dense-core vesicle machinery. *unc-31* encodes a homolog of mammalian CAPS. As CAPS mediate dense-core vesicle (DCV) release of insulin from β-cells in mammals, so does *unc-31* promote DCV release from *C. elegans* neurons (Berwin et al., 1998; Speese et al., 2007). An *unc-31* mutant was found to extend the lifespan of L1 arrested animals in a DAF-16-dependent manner, indicative of reduced IIS (Lee and Ashrafi, 2008). The starvation sensitivity was restored when *unc-31* was re-introduced in ciliated chemosensory neurons, suggesting that food perception and concomitant ILP release require a functional DCV machinery in these cells. Moreover, the same study identified a transient receptor potential vanilloid (TRPV) channel, OCR-1, that likely acted upstream of UNC-31 and was indispensable in these neurons for secretion of the ILP DAF-28. DAF-28 secretion was also shown to be greatly boosted by mutations in *tom-1* and in *bbs* genes. Both *tom-1* and *bbs* genes are negative regulators of DCV release in *C. elegans* ciliated neurons (Lee et al., 2011). Several genes required specifically for DCV were also identified in a clustering analysis (Ch’ng et al., 2008). By comparing various parameters in the presynaptic distribution of fluorescently labelled DCV cargos, small clear synaptic vesicle (SV) cargos, among others, Ch’ng and colleagues were able to identify clusters of genes that likely acted in the same pathways. Specific involvement in DCV release of INS-22/ILP secretion was found in two such clusters. The first was specified by *unc-31* and *unc-36* (a voltage-gated Ca\(^{2+}\) channel subunit), and the second was specified by *egl-8* and *pkc-1*. EGL-8 is known to act through the second messenger DAG, to activate PKC-1, which in turn promotes DCV release (Sieburth et al., 2007).

In addition, (Park et al., 2012) discovered a link between the TGFβ pathway and DAF-28 secretion from ASI neurons. In these neurons, activated (and thus nuclear) DAF-8 binds the nuclear hormone receptor NHR-69 and thereby inhibits expression of the voltage-gated potassium channel EXP-2.
However, when signalling through the TGFβ pathway is low, repression by NHR-69 and DAF-8 is lost. Consequently, EXP-2 levels become high, which attenuates secretion of DAF-28.

**ASNA-1 in IIS and DAF-28 secretion**

Analysis done in *C. elegans* and mammalian cells shows that ASNA-1 is a positive regulator of IIS and insulin secretion (Kao et al., 2007). *C. elegans* animals depleted of asna-1 arrest growth reversibly in the L1 stage. In these animals, a DAF-16::GFP reporter localizes to nuclei, indicative of a reduced signalling strength in the IIS pathway. This conclusion is further supported by several lines of evidence. First, overexpression of ASNA-1, like that of INS-4 and DAF-28, promotes dauer larvae exit in daf-7 mutants but not in daf-2 mutants. Second, daf-7(e1372) mutants are temperature-sensitive and form dauers at 25°C, but very rarely do so at 20°C. However, asna-1(ok938); daf-7(e1372) double mutants do form dauers at 20°C. By contrast, enhancement of dauer formation is not seen in asna-1(ok938); daf-2(e1370) double mutants. Third, since feeding programs are normal, the growth defects are more likely caused by a reduced IIS pathway activity than defective feeding. In addition, ASNA-1 acts non-autonomously and over expression of ASNA-1 from a daf-28 promoter rescues all defects observed in asna-1 mutants. In *C. elegans*, ASNA-1::GFP is expressed in insulin producing cells, such as the ASI neurons and intestinal cells. This expression pattern overlaps with that of a Pdaf-28::DAF28::GFP reporter. Although this reporter is produced at wild type levels in asna-1-depleted animals, its secretion into the pseudocoelomic fluid is severely reduced. Similarly, knockdown of ASNA1 in mammalian cells does not affect synthesis, but rather secretion of insulin. In human pancreas, ASNA1 is expressed selectively in β-cells, consistent with a conserved role in insulin secretion. Moreover, the expression of human ASNA1 in *C. elegans* head neurons rescues growth in asna-1 mutants. These lines of evidence demonstrate a conserved role for ASNA-1 in promoting insulin secretion in both nematodes and mammals.

**Endoplasmic reticulum stress**

The endoplasmic reticulum (ER) forms a tubulated network of membrane-enclosed cisternae, continuous with the nuclear membrane but separate from other organelles. Its functions include membrane synthesis, Ca²⁺ signalling, and protein maturation through glycosylation and folding. The ER has two distinct domains, the rough ER (RER) and the smooth ER (SER). While RER is decorated with ribosomes and participates in protein translocation across the membrane, the lack of ribosomes in smooth ER is
suggestive of functions other than protein targeting. This is reflected by the different composition of the two in different cell types. In *C. elegans*, neurons have more SER and intestinal cells have mostly RER (Rolls et al., 2002). The importance of Ca$^{2+}$ signalling in neurons gives high amount of SER while Ca$^{2+}$ signalling appears less important in intestinal cells, which have almost exclusively RER.

Secreted and transmembrane proteins enter the RER as unfolded polypeptide chains. Because the demands for protein flux into the ER vary, its protein folding capacity needs to be regulated. This is achieved by signalling sensors that respond to increased or decreased folding demands in the ER lumen and convey those signals through transmembrane receptors to cytosolic effectors and transcription factors. These effectors and transcription factors modulate the folding capacity according to the folding demands in the ER to maintain protein homeostasis. This process is generally referred to as the ER unfolded protein response (ER UPR). Defects in the ER UPR that render an inability to maintain protein homeostasis is linked to a number of pathological conditions, including diabetes, neurodegenerative disease and cancer [reviewed in (Hetz, 2012)].

**The ER unfolded protein response**

When there is an imbalance between the load of unfolded proteins in the ER and the capacity of the ER to handle unfolded proteins (i.e. ER stress), a response is triggered that act in three distinct signalling branches to restore ER homeostasis. All branches of this response are essentially conserved from *C. elegans* to humans. These are reviewed below using *C. elegans* nomenclature.

The first branch is defined by the luminal sensor inositol-requiring protein-1, IRE-1/IRE1 (*Figure 4A*). Upon accumulation of unfolded proteins in the ER lumen, IRE-1 oligomerizes and undergoes autophosphorylation (Walter and Ron, 2011), which triggers activity in three directions. First, it splices the mRNA of the transcription factor XBP-1, promoting its translation (Calfon et al., 2002). XBP-1 then promotes transcription of genes that act to restore protein homeostasis (Reimold et al., 2001). Second, IRE-1 activates JNK-1 that in *C. elegans* promotes the nuclear localization of DAF-16/FOXO (Oh et al., 2005). Third, evidence from *Drosophila melanogaster*, showed that IRE1 can also promote cleavage of various mRNAs encoding proteins destined for the ER (Hollien and Weissman, 2006). That way, IRE-1 signalling may act directly to alleviate the protein-folding load on the ER.
In the second branch, the activating transcription factor-6, ATF-6/ATF6, is kept inactive by HSP-4/BiP in the ER lumen (Figure 4C). But since unfolded proteins in the ER lumen competes with ATF-6 for HSP-4 binding, an increased load of unfolded proteins will sequester HSP-4 and hence activate ATF-6/ATF6 (Shen et al., 2002). A cytoplasmic portion of ATF-6/ATF6 is then cleaved (Haze et al., 1999) and further processed in the Golgi apparatus (Shen et al., 2002) to generate an active transcription factor, likely promoting transcription of UPR target genes. In C. elegans, the ATF-6 branch appears important for constitutive UPR regulation during normal development (Shen et al., 2005).

Figure 4. The C. elegans unfolded protein response
IRE-1 (A), PEK-1 (B) and ATF-6 (C) respond to unfolded proteins in the ER lumen by activating transcription of UPR target genes. In parallel, IRE-1-dependent mRNA decay, JNK-1 activation and PEK-1-dependent suppression of general translation also act to reduce ER stress. The figure is adapted and reprinted by permission from Macmillan Publishers Ltd: Nature (Hetz, 2012), copyright (2012).

The third UPR branch involves the protein kinase RNA-like ER kinase, PEK-1/PERK (Figure 4B). Similar to the ATF-6 branch, PEK-1 is kept inactive by binding to HSP-4 but is activated when that bond is lost. The active form of PEK-1 phosphorylates the α-subunit of eukaryotic translation initiation factor-2, eIF2α, which inhibits eIF2 and thus transcription in general. However, a second output of this pathway is present in mammals, where some mRNAs are selectively transcribed under eIF2-inactivated conditions. One of these mRNAs encodes the transcription factor ATF-5/ATF4, which in mammals drives transcription of CHOP. CHOP drives expression of apoptosis-promoting genes (Oyadomari and Mori, 2004). However, C. elegans has no known homolog of CHOP.
Interestingly, the dominant *daf-28(sa191)* mutation induces the ER UPR specifically in DAF-28-expressing intestinal and neuronal cells (Kulalert and Kim, 2013). This is thought to depend on folding problems with mutant DAF-28 protein. The mutation specifically induces the PEK-1/PERK branch of the UPR, which in turn causes constitutive entry into the dauer stage in a mechanism that is partially independent of IIS and TGFβ signalling (Kulalert and Kim, 2013; Malone et al., 1996). Thereby the PEK-1/PERK branch appears to constitute yet another signalling axis controlling the dauer diapause.

**Membrane remodelling during endoplasmic reticulum stress**

During ER stress, signalling through the IRE-1 – XBP-1 axis also promotes synthesis of phospholipids that integrate into the ER membrane, causing an expansion of the ER volume (Shaffer et al., 2004; Sriburi et al., 2004). In yeast it was demonstrated that expansion of the ER lumen during ER stress acts to alleviate ER stress (Schuck et al., 2009). Therefore, lumen expansion is likely a mechanism that either increases the folding capacity or to decreases the sensitivity to unfolded proteins during ER stress.

In addition to all UPR responses described above, misfolded proteins can be retro-translocated to the cytosol, presumably through the SEC61 translocon, ubiquitinated and shuttled for degradation in the proteasome in a process called ER-associated decay (ERAD) [Reviewed in (Goder, 2012)]. However, severely misfolded proteins and aggregated proteins cannot cross the ER membrane to the cytoplasm for subsequent degradation. Under such circumstances, autophagy is triggered. Indeed, many genes mediating autophagy have been shown to be under control of UPR transcription factors (Bernales et al., 2006; Bernales et al., 2007; Ciechomska et al., 2013; Yorimitsu et al., 2006). In ER UPR-induced autophagy, regions of ER are sequestered into autophagosomes, termed ER-containing autophagosomes (ERAs). It was demonstrated that this process acts to alleviate ER stress in yeast and that it protects mouse brains from accumulation of misfolded α-synuclein (Bernales et al., 2006; Steele et al., 2012).

**Mitochondrial function**

Mitochondria are thought to originate from an endosymbiotic relation with a proteobacterium that survived endocytosis in its host, little less than two billion years ago (Emelyanov, 2001). As a remnant of their evolutionary origin, mitochondria are divided into four sub compartments: An outer membrane, separating it from the cytosol, an intermembrane space (IMS), a folded inner membrane and a matrix. The ability of mitochondria to perform
oxidative phosphorylation generated an advantage in the host cells, which had previously depended on glycolysis and fermentation to generate ATP. In animals, mitochondria have besides their major role in ATP production acquired a number of essential functions, such as regulation of apoptosis, calcium signalling, iron-sulfur protein maturation and fat and amino acid metabolism [Reviewed in (Brand et al., 2013)]. Given these important roles, dysfunctional mitochondria are linked to a number of disease conditions such as Parkinson’s disease, Huntington’s disease, Alzheimer’s disease and diabetes (Chaturvedi and Flint Beal, 2013; Maassen et al., 2004).

**Protein import across the outer mitochondrial membrane**

During the course of eukaryotic evolution, the size of the mitochondrial genome has decreased massively, as genes have been either lost or transferred to its host genome (Adams and Palmer, 2003). In animals, the mitochondrial genome encodes less than 40 genes (Wolstenholme, 1992). These are genes for ribosomal RNAs, transfer RNAs and a handful of protein of the mitochondrial respiratory chain (MRC). About 800 to 1500 proteins, depending on species, execute their function in mitochondria, and even the proteins encoded by the mitochondrial genome form functional complexes only in concert with nucleus-encoded proteins (Meisinger et al., 2008; Pagliarini et al., 2008; Reinders et al., 2006; Sickmann et al., 2003). Therefore, correct targeting and import of nucleus-encoded proteins is essential to mitochondrial function.

Nucleus-encoded pre-proteins destined to the mitochondria are generally synthesized on cytosolic ribosomes and targeted post-translationally to the translocase of the outer mitochondrial membrane (TOM) complex (Neupert and Herrmann, 2007) (Figure 5). This complex is composed of core subunits, forming the actual protein-conducting pore and contributing to the stability of the complex, and receptor subunits that bind to targeting sequences of preproteins. Depending on the type of targeting signal, a preprotein is recognized by either TOM70 (internal targeting signals), or jointly by TOM20 and TOM22 receptor subunits (N-terminal cleavable presequences). In a series of subsequent binding steps, which also involves the small subunit TOM5, preproteins are delivered to the pore-forming subunit TOM40. After translocation through TOM40, pre-proteins are sorted to different sub-compartments depending on their targeting signal (Figure 5).

The TOM40 subunit is predicted to have a β-barrel topology, forming the protein-conducting pore of the complex (Hill et al., 1998; Mannella et al., 1996). Therefore it has long been predicted to be incapable of opening
laterally to allow insertion of membrane proteins into the outer membrane. However, this assumption was challenged by a report showing that TOM40 was capable of lateral release of α-helical proteins into the outer membrane (Harner et al., 2011). Still, β-barrel membrane proteins destined to the outer membrane must first transverse the outer membrane through the TOM complex and then insert from the inside through the sorting and assembly machinery (SAM). The only known nucleus-encoded mitochondrial proteins that are not dependent on the TOM complex for correct targeting to the mitochondria are a subset of α-helical membrane proteins in the outer membrane (Meineke et al., 2008; Ross et al., 2009).

In *C. elegans* little is known about TOM proteins. RNAi against *tomm-7/Tom7* affects mitochondrial morphology and TOMM-20 localizes to mitochondrial membranes (Curran et al., 2004). One study showed that RNAi against *tomm-40* may cause mitochondrial fragmentation (Ichishita et al., 2008). However it was speculated that the RFP::TOMM-20 marker used to label mitochondria may not have targeted to mitochondria under TOMM-40-deplete conditions since yeast TOM20 requires TOM40 for correct targeting (Ahting et al., 2005; Ichishita et al., 2008).

![Figure 5. Protein targeting to yeast mitochondria](image)

*Nucleus-encoded proteins are imported across the outer mitochondrial membrane through the TOM complex. They are then sorted, depending on their structure and type of targeting sequence, to different mitochondrial sub compartments by the sorting and assembly machinery, SAM (outer membrane), the translocase of the inner mitochondrial membrane 23, TIM23 (inner membrane and matrix), the translocase of the inner mitochondrial membrane 22, TIM22 (inner membrane) and the mitochondrial intermembrane space assembly, MIA (intermembrane space). Mitochondria-encoded proteins are inserted into the inner membrane by the cytochrome oxidase activity complex (OXA). The figure is adapted and reprinted from (Mossmann et al., 2012) with permission from Elsevier.*
The mitochondrial unfolded protein response in C. elegans

Perturbations that challenge the protein environment in mitochondria, such as high levels of reactive oxygen species (ROS), stoichiometry changes or reduced folding capacity trigger a mitochondrial unfolded protein response (mtUPR). Conceptually, this response appears similar in mammals and in C. elegans. Imbalances are sensed in mitochondria, generating a signal to upregulate nuclear transcription of genes that act to alleviate mitochondrial stress.

In C. elegans, when the load of unfolded proteins exceeds the folding capacity of mitochondrial chaperones, CLPP-1/ClpP protease degrades misfolded proteins to peptides in the matrix (Haynes et al., 2007) (Figure 6). Efflux of these peptides through HAF-1 to the cytosol activates the transcription factor ATFS-1 and promotes UBL-5 binding to DVE-1 (Haynes et al., 2010). Jointly, activated ATFS-1, UBL-5 and DVE-1 promote transcription of the mitochondrial chaperones hsp-60 and hsp-6/mtHsp70. HSP-6 and HSP60 chaperones are then imported into mitochondria, where they capacitate protein folding and alleviate mitochondrial stress (Yoneda et al., 2004).

Figure 6. The mitochondrial unfolded protein response in C. elegans

Misfolded mitochondrial proteins are degraded by ClpP protease to peptides, which are transported out of the matrix through HAF-1. This event activates ATFS-1, UBL-5 and DVE-1, which together promote transcription of hsp-60 and hsp-6/mtHsp70. The figure is reprinted from (Pellegrino et al., 2013) with permission from Elsevier.

Mitochondrial functions in insulin secretion

Mammalian β-cells produce and release insulin in response to elevated levels of blood glucose. The main stimulatory signal for insulin secretion is ATP, mainly generated from oxidative phosphorylation in mitochondria. Oxidative phosphorylation involves the action of five linked protein complexes, electron transport chain (ETC) complex I-V, located in the inner mitochondrial membrane [reviewed in (Wiederkehr and Wollheim, 2006)].
Complex I-IV utilize electrons donated by NADH and FADH2, which are generated in the tricarboxylic acid cycle, to pump protons out from the matrix. This generates a proton gradient (ΔΨ) across the inner membrane. Complex V, the ATP synthase, uses proton influx as the driving force to regenerate of ATP from ADP. ATP is then transported out to the cytosol. In β-cells, elevated levels of cytosolic ATP causes closure of ATP-gated K⁺ channels and depolarization of the plasma membrane. Subsequent opening of voltage-gated Ca²⁺ channels and influx of Ca²⁺ into the cytosol primes dense-core vesicle fusion to the plasma membrane and, hence, exocytosis of insulin. In addition, other stimulatory signals originating in mitochondria can also promote insulin secretion in β-cells. These include intermediates derived from oxaloacetate in the TCA cycle, such as citrate, isocitrate, malate and 2-oxoglutarate (Wiederkehr and Wollheim, 2006).

The flow of electrons through the ETC also generates ROS. These highly reactive substances are either retained in the mitochondrial matrix or released out into the cytoplasm. Both proteins and lipids are susceptible to damage from interactions with ROS. In β-cells, increased ROS levels, induced either by high glucose concentrations or by exposure to hydrogen peroxide, can attenuate insulin secretion (Sakai et al., 2003). On the other hand it was demonstrated that inducing ROS specifically inside mitochondria, with the chemicals antimycin and rotenone, promoted insulin secretion (Leloup et al., 2009), demonstrating that mitochondrial ROS can instead act as a stimulatory signal for insulin secretion.

**Mitochondria in longevity and dietary sensing**

In *C. elegans*, mitochondrial function has a determining role in the regulation of life span. Several pathways that mediate longevity converge in their action to reduce ΔΨ. Mutants in *daf-2/insulin* receptor, as well as mutants in two genes required for ETC function, *clk-1* (Miyadera et al., 2001) and *isp-1* (Feng et al., 2001), all have lowered ΔΨ and increased lifespan (Lemire et al., 2009). However, other mutations affecting the ETC instead have decreased lifespan in spite of lowered ΔΨ. Mutations in *gas-1*, encoding a subunit of ETC complex I and in *mev-1*, encoding a subunit of ETC complex II, both have decreased ΔΨ and decreased lifespan (Brand, 2000; Kayser et al., 2004; Senoo-Matsuda et al., 2001). But when long-lived *daf-2*, *clk-1* and *isp-1* mutants all show decreased generation of ROS (Burgess et al., 2003), *gas-1* and *mev-1* mutants instead have increased ROS production, which may contribute to their reduced lifespan. Indeed, quenching ROS in *mev-1* mutants suppresses their life span reduction (Melov et al., 2000). In addition, the decreased ΔΨ of *daf-2* mutants is dependent on *daf-16*, suggesting that IIS can regulate mitochondrial metabolism (Lemire et al.,...
2009). However, the effect of clk-1 on longevity is independent of daf-2, implying that these two genes act separately to control mitochondrial metabolism and lifespan.

As ΔΨ in several studies was coupled to increased lifespan it should also be noted that ΔΨ is a major driving force for translocation of positively charged presequences into the negatively charged mitochondrial matrix (Figure 5) and (Voos et al., 1999). However, downregulation of several C. elegans genes that are presumably involved in mitochondrial protein targeting, affected larval development without affecting lifespan (Curran et al., 2004). Therefore the possible effects of decreased ΔΨ in promoting longevity in the long-lived mutants mentioned above, may be uncoupled to decreased protein import.

In daf-2 mutants and dietary restricted animals, depletion of the prohibitins PHB-1 and PHB-2, which are located in the inner mitochondrial membrane, was shown to further extend lifespan (Artal-Sanz and Tavernarakis, 2009). At the same time, depletion of PHB-1 and PHB-2 reduced the lifespan of well-fed wild-type animals. These opposite effects were coupled to modulated mitochondrial function and fat metabolism and further highlight the complexity of lifespan regulation and dietary responses in mitochondria.

In addition, an effort to find genes that could alter the transcriptional response to changes in the diet identified 38 C. elegans genes that were important for tuning the expression of genes involved in metabolism (Watson et al., 2013). These genes acted in a transcriptional response system to alter expression profiles in response to changes in diet. Most of these genes encoded mitochondrial proteins that were involved in β-oxidation of fatty acids, the TCA cycle, and amino acid metabolism, further highlighting mitochondria as a major dietary sensor in C. elegans.
METHODS

Most techniques used in this kappa are described in detail in the original papers included. Some of the techniques that are of particular importance are described in greater detail below. The methyl viologen and rotenone treatment protocols are only given here and not elsewhere.

Mitochondrial function assays

Mitochondrial unfolded protein response: A mitochondria-specific unfolded protein response (mtUPR) is triggered in situations where the protein environment of mitochondria is disrupted (Haynes et al., 2007; Haynes et al., 2010; Yoneda et al., 2004). These situations include stoichiometric changes between nucleus-encoded and mitochondria-encoded proteins, excess ROS production and accumulation of unfolded proteins in mitochondria. The mtUPR acts to upregulate expression of hsp-6 and hsp-60. Therefore, transcriptional GFP reporters driven by hsp-6 and hsp-60 promoters can be used to assess the level of mtUPR activity (Yoneda et al., 2004). Animals harbouring the integrated transgenes zcls13 (Phsp-6::gfp) and zcls9 (Phsp-60::GFP) were investigated after feeding RNAi treatment against tomm-40 or after feeding with empty vector control-expressing bacteria. RNAi treated animals were anesthetized in 0.5 mM levamizole and imaged with a Leica DMRB microscope equipped for fluorescence. Identical exposure times were used in all cases.

Mitochondrial protein import: NNT-1 (Nicotinamide Nucleotide Transhydrogenase) is a mitochondrial inner membrane protein involved in proton translocation across the inner membrane. It is predicted by the MitoProt program (Claros and Vincens, 1996) to have a positively charged mitochondrial targeting sequence in its N-terminus and is thus likely to follow the TOMM-TIM23-route of mitochondrial import (Figure 5). A truncated NNT-1::GFP construct spanned the promoter region and the first one and a half exons of NNT-1 (Arkblad et al., 2002). This contained the mitochondrial targeting sequence of NNT-1 but lacked its predicted transmembrane domains. Worms expressing this truncated NNT-1::GFP construct (from the extrachromosomal array svEx127), were treated with tomm-40(RNAi) or empty vector control(RNAi). To assess mitochondrial import of the NNT-1::GFP reporter, RNAi-treated animals were transferred as L4s to agar plates, containing 1.25 mM MitoTracker red CMXRos (Invitrogen, Carlsbad, CA, USA) and seeded with the same bacteria, for 24 hours. Worms were then washed three times in M9, anesthetized in 0.5 mM levamizole and analysed using a Nikon Eclipse C1 confocal microscope.
Mitochondrial membrane potential (ΔΨ): The lipophilic dye tetramethylrodamine ethyl ester (TMRE) passively passes in and out through all biological membranes but is retained more in mitochondria that have high ΔΨ (Loew et al., 1993). Therefore, the level of TMRE staining in cells can be used as an indirect measure of ΔΨ. To investigate the effect of tomm-40(peRNAi) on ΔΨ, animals were hatched onto plates seeded with control or tomm-40(RNAi) bacteria and grown until the L4 stage. Then they were transferred to new RNAi plates containing 30 µM TMRE for 16 hours as described previously (Wiedemann et al., 2004). After three washes in M9, the control(peRNAi) and tomm-40(peRNAi)-treated animals were anesthetized in 0.5 mM levamizole and imaged with a Leica DMRB microscope equipped for fluorescence. Identical exposure times were used in all cases.

Methyl viologen treatment: In cells, methyl viologen acts to induce the formation of reactive oxygen species (ROS), by catalysing redox reactions between electron donors and electron acceptors, such as O₂, thereby causing the formation superoxide radicals. To assess the effects of high but sub-lethal levels of ROS on DAF-28/insulin secretion, slIs69 (DAF-28::GFP) (Kao et al., 2007) animals were grown until their first day of adulthood on plates containing 0.4 mM methyl viologen. They were then anesthetized in 0.5 mM levamizole and analysed with a Leica DMRB fluorescence microscope. In slIs69 animals, DAF-28::GFP is secreted from ASI and ASJ neurons and intestinal cells into the pseudocoelomic fluid, where it is taken up by endocytosing coelomocytes, which lack internal expression of the transgene. The presence of coelomocyte GFP content can therefore be used as a measure of secreted DAF-28::GFP (Kao et al., 2007). In the methyl viologen-treated slIs69 animals, the complete absence or very faint presence of coelomocyte GFP was scored as secretion defective. A minimum of one brightly fluorescent coelomocyte was required to be scored as secretion competent.

Rotenone treatment: Rotenone is a commonly used insecticide that acts by inhibiting electron transfer from the mitochondrial electron transport chain (ETC) complex I to ubiquinone. Its action is well known to induce increased ROS production and allows the study of excess ROS generation in mitochondria. To evaluate the effects of rotenone treatment on DAF-28 secretion in tomm-40(peRNAI) animals, slIs69 (DAF-28::GFP) animals were hatched onto RNAi plates containing 1 µM rotenone in 0.1% DMSO and grown until their first day of adulthood. Worms hatched and grown on RNAi plates supplemented with 0.1% DMSO served as negative control. Secretion competence was scored as above. To assess the effects of rotenone treatment in wild type animals, slIs69 animals were synchronized and grown on
rotenone/DMSO- or DMSO plates as above. Since no untreated svIs69 larva displayed any visible GFP in any coelomocyte at 100x magnification, the presence of visible GFP in any coelomocyte was scored as a secreting larva. However, it is possible that this cut-off is of less utility with other fluorophore excitation setups that may generate stronger GFP fluorescence and hence perhaps visible coelomocyte GFP in untreated svIs69 larvae.

**TA protein targeting assay**

*Construction of markers:* To analyse the ability of tail-anchored (TA) proteins to reach the ER compartment, the canonical TA proteins SEC-61β and CYTB5.1 were tagged with GFP in their N-termini and fused to the intestine-specific vha-6 promoter. Similarly, to label the ER compartment, the well-characterized ER membrane protein SP12 was given an N-terminal mCherry tag and fused to the same promoter. Lines expressing mCh::SP12 together with either GFP:: SEC-61β or GFP::CYTB5.1 were generated by co-injection of the markers (all at 50 ng/µl). SEC-61β gave svEx900 (Pvha-6::gfp::sec-61β; mCh::SP12) and CYTB5.1 gave svEx917 (Pvha-6::gfp::cytb5.1; mCh::SP12). The integrated transgene svIs135 (Pvha-6::gfp::sec-61β; mCh::SP12) was generated by exposing svEx900 animals to 40 Grays of irradiation. To get rid of background mutations, svIs135 was backcrossed six times to the wild type N2 strain.

*Reverse genetics:* To make dsRNA for injection RNAi against wrb-1, the full-length cDNA of wrb-1 was cloned into the L4440 vector as a HindIII/HindIII fragment and in vitro transcribed using a commercially available kit (Life Technologies, Waltham, Massachusetts, USA). To make dsRNA against cee-1 and sgt-1, plasmids isolated from an RNAi clone library (Fraser et al., 2000) were in vitro transcribed using the same kit as above. The cee-1 clone was sequenced and confirmed to correspond to cee-1. dsRNA corresponding to wrb-1, cee-1 or sgt-1 was injected into the gonads of svIs135-expressing adult mothers. Injected mothers were transferred to new plates every 24 hours to separate the offspring into different windows. Offspring from the second window generally gave the strongest effects and was analysed as adults for TA protein targeting capacity.

*Confocal analysis:* Adult animals of different genetic backgrounds and with different treatments were analysed with a Nikon A1 confocal microscope. By capturing images with 1 µm intervals throughout the full width of the int1 and int2 intestinal cell rings, stacks of images corresponding roughly to the anterior-most 6 intestinal cells were collected for each animal. Each image was analysed for the presence of GFP foci that were not co-localizing with mCherry. This was done after image acquisition by increasing the intensity of
the red channel in each image to see if a given GFP structure had corresponding mCherry staining. An animal that had any GFP::SEC-61β foci that was completely devoid of corresponding mCherry::SP12 was scored as TA protein targeting deficient. By this method, 0% of the svIs135 animals were scored as such. The number of analysed animals for each condition is indicated in brackets: svIs135 [31], svIs135; asna-1(ok938) [19] svIs135; wrb-i(tm5938) [12] svIs135; wrb-i(RNAi) [16], svIs135; cee-i(RNAi) [10] svIs135; sgt-i(RNAi) [8] suEx917 [13] suEx917; asna-1(ok938) [7], svIs135; starvation [8], svIs135; 10 mM DTT [19].

Electron microscopy

Transmission electron microscopy (TEM): Worms were grown until the first day of adulthood and then washed three times in M9. Immediately after transfer to a fixative solution, containing 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate, an anterior portion of the head was cut off with a scalpel to ensure a fast infiltration of the fixative. The samples were then kept overnight at 4 °C. After this step the samples were further processed by the EM core facility at Umeå University: Samples were washed three times in fixative solution and post-fixed with 1% (v/v) osmium tetroxide for 1 h, followed by two washes in distilled water. They were dehydrated with 50, 70, 95, 100% ethanol and infiltrated and embedded in Spurr’s resin. Using a Diatome diamond knife on a Leica EM UC7, thin sections (60–90 nm) were collected onto copper grids, then treated with 5% uranyl acetate in water for 20 min followed by Sato’s lead staining for 5 min. Sections were examined (by me) with a Jeol 1230 transmission electron microscope. Images were captured using a Gatan MSC 600CW camera.

Immuno-electron microscopy (Immuno-EM): Worms were harvested as one-day adults and washed in M9 as above but instead decapitated in 2% PFA. After embedment of animals 4 by 4 in agarose blocks, they were put back in 2% PFA and immediately handed over to the EM core facility for subsequent processing: Samples were then first fixed in 4% PFA for 4 hours. They were then rinsed with 0.15% glycine in 0.1 M phosphate buffer, pelleted in 10% gelatine/phosphate buffer and cryo-protected by infusion of a 2.3 M sucrose/phosphate buffer overnight at 4°C. 1 mm³ tissue blocks were mounted onto specimen holders and flash frozen in liquid nitrogen. Ultrathin cryosections (70–90 nm) were cut with a Diatome diamond knife at -110°C using a Leica Ultracut UCT with an EM FCS cryo-attachment (Leica, Wetzlar, Germany). Sections were picked up in a 1:1 mixture of 2.3 M sucrose and 2% methyl cellulose as previously described (Liou et al., 1996), and transferred onto Formvar/carbon-coated copper grids. Immuno-labelling was essentially performed as previously described (Tokuyasu,
1980): Grids were placed on 2% gelatine at 37°C for 20 minutes and then rinsed with 0.15% glycine/PBS. The grids were blocked overnight with 1% cold-water fish-skin gelatine and then incubated for 1 hour at room temperature with previously titrated 1C51 primary mouse anti-mCherry antibody (Abcam, Cambridge, UK), diluted to 1:250 x in 1% BSA/PBS. After washing, sections were incubated with secondary goat anti-mouse IgG (BBI, England), diluted 1:20 in 1% BSA/PBS, for 1 hour at room temperature. Sections were then rinsed for 10 minutes in PBS, for 5 minutes in water and then stained on ice for 10 minutes with 0.4% uranyl acetate in 1.8% methylcellulose. Specimens were then examined (by me) using the same equipment as above.
AIMS

The overall aim of this thesis is to gain a better understanding of insulin/IGF signalling (IIS), insulin secretion and the function of ASNA-1, using *C. elegans* as a model system.

Specific aims:

1. Identify new regulators of IIS and insulin secretion based on the *asna-1* phenotype in *C. elegans*.

2. Investigate one of the identified IIS regulators in more detail and explore its functions in mitochondria, in IIS and in insulin secretion.

3. Set up an *in vivo* system to study the role of ASNA-1 and its potential receptor WRB-1 in TA protein targeting to the ER.

4. Explore the ultrastructure of cells in *asna-1* and *wrb-1* mutants.
RESULTS

Identification of new genes that modify insulin signalling

*Caenorhabditis elegans* regulators of IIS have mostly been identified in screens for increased longevity or defective regulation of the dauer diapause. However, *asna-1* is a positive regulator of IIS and DAF-28/insulin secretion (Kao et al., 2007) that has not been identified in such screens, likely because its inactivation does not cause increased longevity or defects in dauer formation by itself upon inactivation. Instead, a strong inactivation of *asna-1* causes a larval arrest at the L1 stage, before the L2/D dauer commitment stage is reached. Also, maternally rescued *asna-1* mutants are pale, scrawny, small and sterile, and do not have increased lifespans. Therefore it seemed possible that a set of IIS regulators could be found that shared the *asna-1* phenotype.

To find new regulators of IIS, we therefore screened a set of potential *asna-1* interactors for *asna-1*-like phe

otypes.

A restricted set of genes was selected based on predicted or confirmed interactions with *asna-1* homologs in various organisms. Genome-wide predictions, large-scale yeast two hybrid studies, co-immunoprecipitation studies and suppressor/enhancer screens have identified of 77 genes that interact with the yeast homologue Get3 (Collins et al., 2007; Ito et al., 2001; Krogan et al., 2006), 6 genes that interact with the *Drosophila melanogaster* homologue CG1598 (Giot et al., 2003), and 13 genes that interact with *C. elegans* *asna-1* (Li et al., 2004; Zhong and Sternberg, 2006). Using a combination of BLASTp and SMART (Letunic et al., 2006) programs, we identified potential *C. elegans* homologs based on primary sequence comparisons and domain structure comparisons. From this final list of 143 genes, feeding RNAi was performed against 86 genes in the RNAi hypersensitive strain *rrf-3(pk1426)*. RNAi clones that produced scrawny and pale larvae that did not grow past the larval stage L3 after 4 days at 20°C were selected for further analysis. This phenotype represents a weak *asna-1* phenotype, reminiscent of feeding RNAi against *asna-1*. The genes identified using these criteria were *iars-1* (isoleucyl tRNA synthase), *rps-0* (cytoplasmic ribosomal protein), *ykt-6* (v-SNARE) and *tomm-40* (translocase of the outer mitochondrial membrane) (Figure 7A). An additional set of clones (*enpl-1, goa-1, mrps-2*) that affected growth is described elsewhere (Natarajan, 2012).

Hypothetically, defects in feeding could produce slow growth. But since *asna-1*-depleted animals have growth defects without associated feeding

---

4 The entire list of genes is found in the online supplementary material for paper 1.
defects we wished to eliminate genes, which upon RNAi knockdown could have caused defective growth because of feeding defects. Feeding abilities were investigated in three ways. First, the rate of feeding was quantified by measuring pharyngeal pumping rates. Second, the ability to ingest bacteria-sized fluorescent beads was assayed. And third, the ability of intestinal cells to internalize luminal content, by means of endocytosis of the fluorescent dye FM4-64 was tested. RNAi against iars-1, rps-0, ykt-6 or tomm-40 had little or no effect on feeding as measured by these assays (Paper II).

**Figure 7. daf-16 suppresses growth defects of ykt-6 and tomm-40.**

A. Fraction of animals above larval stage L3 after 4 days of the indicated feeding RNAi treatment.

B. Changes in growth rates with the daf-16(mgDf50) mutation. RNAi treatments are indicated. Growth rate was defined as the fraction of animals growing to above stage L3 after 4 days of RNAi treatment. The growth rate coefficient can be interpreted as the slope of the line drawn between the mean growth rate of otherwise wild-type animals and the mean growth rate of daf-16(mgDf50) mutants, where 0 corresponds to a horizontal line and no change in growth rate. 1 corresponds to a twice as large fraction reaching above stage L3 in the daf-16(mgDf50) background etc. Error bars represent +/- SE for two individual experiments. *** P<0.001, NS=not significant.

Since no feeding defects were observed that could account for the growth defects, the possibility that the growth defects instead were due to a reduced insulin/IGF signalling (IIS) was investigated. The daf-16(mgDf50) mutation mimics the effect of high IIS, since high IIS acts to inactivate DAF-16 by preventing it from entering nuclei (Henderson and Johnson, 2001). The candidate genes were knocked down with RNAi in the daf-16(mgDf50)
mutant background and the fraction of animals that grew beyond stage L3 after 4 days was scored. The growth defects of iars-1(RNAi) and rps-0(RNAi) animals were not daf-16-dependent (Figure 7B). However, the growth defects of both ykt-6(RNAi) and tomm-40(RNAi) animals was suppressed by daf-16(mgDf50), indicating that growth in these animals might be partially dependent on signalling through the IIS pathway. Consistently, a DAF-16::GFP marker was cytoplasmic in iars-1(RNAi) animals, but nuclear in both ykt-6(RNAi) and tomm-40(RNAi) animals, further supporting the idea that IIS was reduced (Figure 8). Unexpectedly, DAF-16::GFP also appeared in nuclei of rps-0(RNAi) animals in spite of their lack of growth defect suppression by daf-16(mgDf50). If weakened IIS was the cause of the slow growth in rps-0(RNAi) it would be expected the growth defect would be suppressed by daf-16. The nuclear localization of DAF-16::GFP in these animals could be due to changes in signalling through other pathways than the IIS pathway. Indeed, several other pathways converge on DAF-16 to promote or inhibit its nuclear translocation.

![Figure 8](image)

Figure 8. RNAi against rps-0, ykt-6 and tomm-40 causes translocation of DAF-16 to nuclei.
Fluorescence micrographs of stage-matched animals showing the distribution of a DAF-16::GFP marker after indicated RNAi treatment. Arrows point to nuclei devoid of DAF-16::GFP. Arrowheads point to nuclear DAF-16::GFP.

The IIS pathway acts synergistically with the TGFβ pathway in controlling entry into the dauer stage. Therefore, knockdown of an IIS-promoting gene is predicted to act synergistically for dauer formation with a mutation that weakens TGFβ signalling but not with a mutation that weakens IIS (Kao et al., 2007). This prediction was tested in the investigation of TOMM-40. tomm-40(RNAi) acted synergistically with the temperature-sensitive daf-7(e1372) mutation to produce dauers at 20°C (Paper II). 42% (n=124) of the daf-7(e1372); tomm-40(RNAi) animals transiently entered a dauer-like
stage at this temperature, whereas this was never observed in (n=74) daf-7(e1372); control(RNAi) animals (Paper I). Conversely, tomm-40(RNAi) did not enhance dauer formation in temperature-sensitive daf-2(e1370) mutants (n>100) at 15°C. These two experiments suggested that reduced levels of TOMM-40 caused a reduced strength of signalling in the IIS pathway rather than in the TGFβ pathway. Also, since the daf-7(e1372); tomm-40(RNAi) animals did not become arrested in the dauer-like stage it is possible that factors preventing dauer entry were affected, rather than factors promoting dauer exit. One such factor that prevents dauer entry is secretion of the insulin-like peptide DAF-28 (Cornils et al., 2011).

*ykt-6 and tomm-40 are positive modulators of DAF-28/insulin secretion*

DAF-28 promotes reproductive growth and likely acts as an agonistic ligand to the DAF-2 receptor (Li et al., 2003). To test if knockdown of the identified genes caused a defect in the secretion of DAF-28 we made use of a strain harbouring the integrated transgene suIs69 (Pdaf-28::daf-28::gfp). This strain expresses the DAF-28::GFP protein under control of the daf-28 promoter, which is active in ASI and ASJ neurons and in intestinal cells. From these cells, DAF-28::GFP is secreted into the pseudocoelomic fluid, from where it is taken up by coelomocytes (Kao et al., 2007). Since coelomocytes do not express this transgene, any DAF-28::GFP that accumulates results from uptake of secreted protein. The kinetics of DAF-28::GFP secretion only allows detection of GFP in coelomocytes of adult, otherwise wild type animals. But detection is not possible in larvae, presumably because not enough DAF-28::GFP is secreted during the larval development. Consequently, the RNAi-treated animals had to be analysed as adults. With conventional feeding RNAi treatment against tomm-40, worms never reached adulthood. To obtain a weaker RNAi effect, suIs69 embryos were hatched onto plates seeded with RNAi bacteria. This post-embryonic RNAi (peRNAi) presumably allowed expression of wild-type TOMM-40 protein during the entire embryonic development and generated less affected animals that grew up to adulthood. In suIs69-expressing animals, RNAi against iars-1 and rps-0 had no or little effect on secretion of DAF-28::GFP (Figure 9A). However, secretion of DAF-28::GFP was dramatically reduced upon both ykt-6(RNAi) and tomm-40(peRNAi).
Figure 9. RNAi against ykt-6 and peRNAi against tomm-40 diminishes DAF-28::GFP secretion

A. The fraction of adult animals competent of secreting DAF-28::GFP upon the indicated RNAi regimen. Animals containing a minimum of one brightly fluorescing coelomocyte were scored as secretion competent. Error bars show +/- SE for three independent RNAi experiments for lars-1, rps-0 and ykt-6 and include one additional independent RNAi experiment for control and tomm-40(peRNAi).

B. Paired DIC and fluorescence micrographs of a secretion-defective ykt-6(RNAi) animal showing granular accumulation pattern of DAF-28::GFP around the distal, posterior gonad arm (top panels) and in a posterior intestinal cell (bottom panels). Arrows indicate the posterior-anterior axis, n=nucleus.

C. Over expression of DAF-28 restores the cytoplasmic retention of DAF-16 in tomm-40(RNAi) animals. Fluorescence micrographs of two tomm-40(RNAi) treated siblings, carrying an integrated DAF-16::GFP transgene. A co-injected coelomocyte RFP marker labelled the presence of the extrachromosomal Pdaf-28::daf-28 array (red arrows) in the right panel. White arrowheads indicate nuclear DAF-16::GFP and white arrows indicate nuclei that are devoid of DAF-16::GFP.

Defective uptake of secreted proteins by coelomocytes or general secretory defects could potentially cause the decreased DAF-28::GFP secretion seen in ykt-6(RNAi) and tomm-40(peRNAi) animals. Therefore we tested secretion and GFP protein uptake by coelomocytes in a strain harbouring the arls37 transgene (Fares and Greenwald, 2001), which encodes a signal sequence-GFP fusion (gfp), driven by the myo-3 promoter. In this strain, ssGFP is
synthesized in body wall muscle cells, secreted into the pseudocoelom and taken up by coelomocytes. However, no decreased coelomocyte GFP content was detected under any of the above RNAi regimens (Paper I and Paper II). Therefore the reduced DAF-28::GFP secretion in ykt-6(RNAi) and tomm-40(peRNAi) animals is not due to general secretion defects or coelomocyte uptake defects. In addition, none of the assayed svIs69 animals had any detectable decreases in neuronal expression of the DAF-28::GFP transgene (Paper I and Paper II), indicating that the low amounts of secreted DAF-28::GFP protein in ykt-6(RNAi) and tomm-40(peRNAi) animals was not likely due to decreased DAF-28::GFP protein synthesis. Intriguingly, in secretion-defective ykt-6(RNAi) animals the DAF-28::GFP protein instead accumulated beyond the levels of controls. This accumulation appeared granular in nature and was predominantly found in posterior intestinal cells and in gonadal sheath cells (Figure 9B). Accumulation, or even localization of DAF-28 in gonadal sheath is previously unreported.

Since growth-defective tomm-40(RNAi) animals had reduced DAF-28::GFP secretion (Figure 9A) and reduced IIS (Figure 7B, 8 and daf-7 dauer enhancement experiments), it was investigated whether the IIS strength could be restored by over expression of DAF-28. To do that, a line carrying both the integrated daf-16::gfp transgene and an extra-chromosomal high copy Pdaf-28::daf-28 transgene for DAF-28 over expression was generated. Because extrachromosomal arrays are unstable, only some of the progeny overexpressed DAF-28. In siblings treated with tomm-40(RNAi) on the same culture plate, 70.6% (n=17) of the animals without the Pdaf-28::daf-28 transgene showed nuclear localization of DAF-16::GFP, while only 14.8% (n=27) of the animals with the transgene did so (Figure 9C). This indicated that overexpression of DAF-28 could bypass the IIS defect in tomm-40(RNAi) animals.

Both an ANF::GFP transgene (Speese et al., 2007) and an INS-22::VENUS transgene (Ch’ng et al., 2008) are expressed in neurons and undergo dense-core vesicle-dependent secretion into the pseudocoelom. In a manner similar to DAF-28::GFP, marker uptake by coelomocytes can be used to measure levels of secreted protein (Ch’ng et al., 2008; Speese et al., 2007). Upon treatment with tomm-40(peRNAi), we observed no decreased neuronal secretion of either ANF::GFP or INS-22::VENUS (Paper II). Since DAF-28 is also secreted from dense-core vesicles, we were in this way able to discriminate between the secretion patterns of different dense-core vesicle cargos, based on their dependence on tomm-40.
Since TOMM-40-depleted animals displayed reduced IIS, which at least in part was due to defective DAF-28 secretion, we undertook a detailed analysis of tomm-40 gene function in hope of finding possible causes of these defects.

TOMM-40 is a ubiquitously expressed mitochondrial protein

Amino acid sequence comparisons of *C. elegans*, *S. cerevisiae*, *D. melanogaster* and human versions of TOMM-40, showed a high degree of conservation across phyla. *C. elegans* TOMM-40 shares 49% and 42% sequence homology with fly and human versions respectively. The yeast version of the gene encodes a core subunit of the translocase of the outer mitochondrial membrane (Pfanner and Geissler, 2001). Consistent with a predicted role as a mitochondrial translocase, transcriptional and translational reporter analysis revealed that *C. elegans* TOMM-40 was ubiquitously expressed and localized mitochondria (Figure 10A). Ring-like localization of TOMM-40::GFP encircled MitoTracker Red foci, which are known to represent the mitochondrial matrix (Figure 10B). This indicated that TOMM-40::GFP likely localized to mitochondrial membranes, which is in keeping with its predicted role as a mitochondrial translocase subunit.
Figure 10. TOMM-40 is ubiquitously expressed and targets to mitochondria.

AD. Fluorescent and paired DIC images of an animal expressing a Ptomm-40::GFP transgene. Expression was detected in (A) the nerve ring (arrow), pharyngeal muscle (arrowhead), (B) gonadal sheath cells (arrow), (C) intestinal cells (arrow) and (D) tail hypodermal cells (arrow).

E. Confocal images of a body wall muscle cell expressing a TOMM-40::GFP transcriptional fusion protein and stained with MitoTracker red. Arrowheads indicate the localization of TOMM-40::GFP in ring-like structures that encircle MitoTracker foci.

TOMM-40 is a mitochondrial translocase required for mitochondrial function

The homologs of TOMM-40 in other organisms are required for transport of nucleus-encoded proteins into mitochondria [reviewed in (Neupert and Herrmann, 2007)]. To test the role of C. elegans TOMM-40 in mitochondrial protein transport we utilized a transgene carrying a truncated version of nnt-1 fused to GFP. NNT-1 is a nucleus-encoded protein that has a predicted mitochondrial targeting sequence in its first exon (Paper II). The nnt-1::gfp construct spanned the promoter region of nnt-1 and the first one and a half exons of its open reading frame (Arkblad et al., 2002). In control RNAi animals, this truncated NNT-1::GFP protein was expressed in intestinal cells, where it co-localized with MitoTracker Red in mitochondrial tubules (Figure 11A). However, tomm-40(RNAi) treatment abolished the co-
localization between MitoTracker Red and NNT-1::GFP. The NNT-1::GFP fluorescence instead became diffusely dispersed in the cytoplasm, indicating that mitochondrial protein targeting was defective in tomm-40(RNAi) animals. Also, the aggregated, non-tubular MitoTracker Red staining suggested that mitochondrial distribution or morphology was altered by tomm-40(RNAi).

Figure 11. TOMM-40 is a translocase required for mitochondrial function

A. Confocal micrographs of intestinal cells stained with MitoTracker Red and expressing the truncated mitochondrial protein probe NNT-1::GFP. RNAi treatment is indicated. Arrows point to tubular mitochondria in a control animal, where NNT-1::GFP and MitoTracker Red co-localize. Scale bars are 20 µm.

B. Fluorescence micrographs of RNAi-treated animals expressing transcriptional stress markers or stained with TMRE. Exposure times are indicated.

Since mitochondrial protein import was defective in tomm-40(RNAi) animals, we reasoned that other mitochondrial functions might also have been affected. Perturbations of mitochondrial protein homeostasis are known to trigger a mitochondrial unfolded protein response (Yoneda et al., 2004). HSP-6 and HSP-60 are chaperone effectors in this response and are upregulated upon insufficient folding capacity or stoichiometric imbalances in mitochondria. We observed a strong upregulation in both Phsp-6::GFP and Phsp-60::GFP transcriptional reporters upon tomm-40(RNAi) (Figure 11B). In addition, the Phsp-6::GFP reporter showed a robust upregulation in the tomm-40(tm4574) mutant (supplementary information to Paper II). However, no upregulation of the ER stress marker Phsp-4::GFP (Calfon et al., 2002), the cytosolic stress marker Phsp-16.2::GFP (Link et al., 1999) or the oxidative stress marker Pgst-4::GFP (Leiers et al., 2003) could be detected upon tomm-40(RNAi) (Paper II). Nor was there any evidence of oxidative protein damage using a commercially available OxyBlot kit (Paper...
II). Therefore, the defects in growth and IIS associated with the lack of TOMM-40 are not likely due to ER-, cytosolic- or oxidative stresses.

During oxidative phosphorylation, mitochondria generate an electrochemical potential (ΔΨ) across the inner mitochondrial membrane. ΔΨ is used by the ATP synthase to regenerate ATP from ADP. The dye tetramethylrodamine ester (TMRE) passively passes through biological membranes, but is retained in mitochondria to a higher degree when ΔΨ is high (Scaduto and Grotjohann, 1999). Therefore, the levels of TMRE staining can be used to compare levels of ΔΨ. We observed substantially less TMRE staining in tomm-40(RNAi)-treated animals compared to controls (Figure 11B), indicating a collapse of the ΔΨ in these animals.

In summary, mitochondrial function was severely compromised in animals lacking TOMM-40, since protein import was reduced, the mitochondrial protein milieu was disrupted, and since ΔΨ had collapsed. We propose that these defects may cause reduced DAF-28 secretion (Figure 9A) that in turn reduces IIS (Figure 9C), resulting in retarded growth (Figure 7A) in TOMM-40-depleted animals.

Low levels of reactive oxygen species in mitochondria stimulate DAF-28/insulin secretion.

It is known that various forms of mitochondrial perturbations can generate elevated levels of reactive oxygen species (ROS). It is also known that high levels of ROS causes reduced insulin secretion from mammalian β-cells (Krippeit-Drews et al., 1999; Maechler et al., 1999; Sakai et al., 2003). On the other hand, low levels of ROS generated within mitochondria during oxidative phosphorylation can instead act as a stimulus for glucose-induced insulin secretion in rat islets (Leloup et al., 2009). We tested whether these two oppositely acting consequences of ROS had an impact on DAF-28 secretion in wild type and tomm-40-depleted worms.

Worms expressing DAF-28::GFP were exposed to high levels of ROS both genetically and pharmacologically. First, we used the genetic mutant mev-1(kn1), which is defective in a subunit of complex II in the mitochondrial electron transport chain. mev-1 mutants have high levels of ROS and are short lived (Ishii et al., 1998; Senoo-Matsuda et al., 2001). However, these animals have normal ATP levels due to a compensatory increase in cytosolic ATP generation via the fermentative pathway. This suggests that the effects of high ROS can be studied without the confounding effect of lowered ATP levels. We found that mev-1 mutants had significantly lower levels of DAF-28::GFP secretion (Figure 12A). This is consistent with a previous report,
showing that DAF-16::GFP is nuclear in this mutant (Kondo et al., 2005). Second, to pharmacologically induce ROS, worms expressing DAF-28::GFP were exposed to a high, but sub-lethal, (0.4 mM) concentration of methyl viologen, which is a non-specific ROS inducer. Control(RNAi) animals exposed to 0.4 mM methyl viologen displayed reduced levels of secreted DAF-28::GFP (Figure 12A). However, many worms exposed to methyl viologen never reached adulthood, but instead arrested in a dauer-like state, much similar to the one that was transiently entered in daf-7(e1372); tomm-40(RNAi) animals (Paper II).

Figure 12. Dual effects of reactive oxygen species on DAF-28::GFP secretion

A. Fraction of adults competent of secreting DAF-28::GFP after the indicated treatment. Animals containing a minimum of one brightly fluorescing coelomocyte were scored as secretion competent. Error bars for tomm-40(RNAi); DMSO and tomm-40(RNAi); 1 µM rotenone show +/- SE for two independent experiments. For the other conditions, sample sizes are indicated as n-numbers.

B. Fraction of larvae competent of secreting DAF-28::GFP. Any visible GFP in a coelomocyte was scored as a secreting larva. Sample sizes are indicated as n-numbers.

The insecticide rotenone induces ROS specifically in mitochondria by changing the structural conformation of electron transport chain complex I (Ravanel et al., 1984). We attempted to establish low levels of mitochondrial ROS by using 1 µM rotenone, which is 1/750th of its LD50 for C. elegans (Ishiguro et al., 2001). Under these conditions, the secretion defect of tomm-40(peRNAi) animals appeared to be partially suppressed (Fig. 7A). The effect of rotenone was also assessed independently of tomm-40 manipulation. For this we took advantage of the DAF-28::GFP secretion kinetics in suls69 animals: Secreted DAF-28::GFP is only detectable in coelomocytes of adult animals, but never in larvae. Strikingly, DAF-28::GFP was readily seen in coelomocytes of larvae upon rotenone treatment, indicating enhanced secretion (Figure 12B). Taken together these results support the notion
that, just like insulin secretion in mammalian β-cells, DAF-28 secretion is inhibited by high levels of unspecific ROS but stimulated by low levels of mitochondrial ROS.

**asna-1 promotes ER-targeting of the tail-anchored protein SEC-61β in vivo**

Work done in reconstituted mammalian systems and in yeast has shown that ASNA1/Get3 promotes targeting of tail-anchored (TA) proteins to the ER membrane (Schuldiner et al., 2008; Stefanovic and Hegde, 2007). However, these studies have not investigated TA protein targeting in the context of a genetically tractable, multicellular organism. Hence, the effect of this process on physiology and metabolism remains unknown. In *C. elegans*, *asna-1*-depleted animals present a wide array of phenotypes and it is currently unclear if all of these phenotypes are consequences of defective TA protein targeting. Therefore, we reasoned that investigations of TA protein targeting in *C. elegans* could present opportunities to 1) discriminate between possible TA protein-dependent and TA protein-independent functions of *asna-1* and 2) study the process of TA protein targeting in regulation of physiology and metabolism. A necessary first step in this analysis would be to set up an assay system for TA protein targeting in *C. elegans*.

To set up such a system, GFP tagged model TA proteins were co-expressed with mCherry-tagged SP12 (mCh::SP12), which is an established marker to label the ER membrane in *C. elegans* (Rolls et al., 2002). The fusion proteins were expressed using the *vha-6* promoter, which is active only in the intestine. This promoter was chosen because intestinal cells are big and easy to visualize and because intestinal cells express *asna-1* (Kao et al., 2007). In biochemical studies, the TA protein Sec61β has been shown to require ASNA1 for correct targeting to the ER membrane, while another TA protein, Cytochrome B5 (CytB5), is targeted to ER membranes independently of ASNA1 (Favaloro et al., 2008; Stefanovic and Hegde, 2007). The worm homologs of these two proteins, SEC-61β (Y38F2AR.9) and CYTB5.1, fulfil the criteria for TA proteins (Kalbfleisch et al., 2007) because they lack a signal sequence and have a single transmembrane domain very close to the C-terminus, as predicted by the Phobius program (Kall et al., 2004). To test if targeting of these two TA proteins to the ER depended on *asna-1* we compared their localization patterns in live animals using confocal microscopy. In otherwise wild-type animals, both GFP::SEC-61β and GFP::CYTB5.1 co-localized completely with the mCh::SP12 marker, indicating that they were efficiently targeted to the ER (**Figure 13A**). However, in *asna-1(ok938)* mutants GFP::SEC-61β no longer co-localized with mCh::SP12. Instead, GFP::SEC-61β accumulated in foci that did not
overlap with mCh::SP12, indicating non-ER localization of GFP::SEC-61β (Figure 13A). By contrast, GFP::CYTB5.1 never accumulated in mCherry-negative foci in asna-1 mutants, which indicated that GFP::CYTB5.1 was still able to reach the ER in these animals (Figure 13B).

The pattern of mCh::SP12 fluorescence itself appeared more condensed and agglomerated compared to wild type, suggesting a remodelling of the ER membrane itself or a re-distribution of the mCh::SP12 marker to non-ER compartments. This raised the concern that the mCh::SP12 protein may not have been a suitable marker for the ER membrane. To address this, we first tested co-localization with between mCh::SP12 and GFP::KDEL, which is another well-studied marker for the ER. In otherwise wild-type animals, these two markers co-localized completely (Paper III). Second, the ability of the mCh::SP12 marker to reach ER membranes in wild type and asna-1(ok938) animals was studied by immuno-EM. On grids with svIs135 sections, the ER membrane was not successfully identified. This could be due to a failure in treating these sections with contrasting agents. Nevertheless, the mCh::SP12 marker was found adjacent to bright, reticular patches that most likely correspond to ER lumen (Figure 13C). However, in svIs135 (gfp::sec-61β; mCh::SP12); asna-1(ok938) animals, the same marker was found adjacent to membranes in all areas where the ER membrane could be successfully identified. Hence the mCh::SP12 marker appeared to successfully decorate the ER even in ASNA-1-deplete conditions. In addition, the levels of mCherry::SP12 staining was substantially lower in svIs135; asna-1(ok938) animals, as indicated by a much lower density of gold particles. This was consistent with a visual inspection of the fluorescent markers using a dissection microscope. Both GFP and mCherry levels were always lower in asna-1(-/-) homozygotes compared to asna-1(-/+) heterozygotes.
Figure 13. ASNA-1 is required for targeting of SEC-61β to the ER in vivo

A. Confocal micrographs of svIs135 animals co-expressing GFP::SEC-61β and mCh::SP12. The white arrows indicate GFP::SEC-61β foci that do not co-localize with the mCh::SP12 marker. White, dashed squares indicate the areas that are enlarged in the right most panels. Scale bars are 20 µm.

B. Confocal micrographs of animals co-expressing GFP::CYTB5.1 and mCh::SP12. White arrowheads indicate a GFP::CYTB5.1 foci that overlaps with (weaker) mCh::SP12 staining. White, dashed squares indicate the areas enlarged in the right most panels. Scale bars are 20 µm.

C. Transmission electron microscopy images, visualizing immuno-gold labelling of mCherry antibodies in an svIs135 animal (left panel) and an svIs135; asna-1(ok938) animal (right panel). In svIs135 alone, detection of ER membranes failed, but localization of gold particles specifically to the border between dark (electron dense) patches and bright, reticular patches is suggestive of ER membrane localization (arrowhead). In svIs135; asna-1(ok938) gold particles were detected in close vicinity of the ER membrane (arrow).
**wrb-1 has partially overlapping phenotypes with asna-1**

Mammalian WRB is an ER-resident receptor subunit for ASNA1 that participates in ASNA1-dependent TA protein targeting to the ER in *vitro* (Vilardi et al., 2011). WRB also rescues growth and TA targeting deficiencies in yeast Get1; Get2 double mutants (Vilardi et al., 2011; Vilardi et al., 2014). The *C. elegans* WRB-1 protein shares 24% identical primary sequence with mammalian WRB. In addition, WRB, Get1 and WRB-1 all have similar secondary structures, with a three transmembrane domain topology and a predicted (WRB) (Vilardi et al., 2011), confirmed (Get1) (Mariappan et al., 2011) or weakly predicted (WRB-1) (Paper III) coiled-coil domain between the 1st and 2nd transmembrane domains. The coiled-coil domains of Get1 and WRB are required for their interactions with Get3 and ASNA1 respectively. Because of its predicted domain architecture and its structural resemblance with WRB, WRB-1 appeared as a good candidate ER receptor for ASNA-1 in *C. elegans*. We therefore tested the prediction that WRB-1 is an ER-resident protein that is required for TA protein targeting to the ER in *C. elegans*.

First, a GFP::WRB-1 fusion protein driven by an intestinal-specific promoter was investigated. This fusion protein was found to partially localize to the ER (Paper III). Second, to investigate TA protein targeting to the ER, the same svIs135 (GFP::SEC-61β; mCh::SP12)-expressing animals as described above were used. wrb-1(RNAi) affected ER targeting of GFP::SEC-61β, which resulted in its aggregation in foci that were negative for mCh:SP12 fluorescence (Figure 14A). Thus, it appeared that WRB-1 functions in TA protein targeting to the ER, just like its mammalian homolog. We hypothesized that if WRB-1 were a receptor for ASNA-1 in TA protein targeting, wrb-1 deletion mutants would likely have overlapping phenotypes with those of asna-1 mutants. Therefore, we compared the TA protein targeting and morphology phenotypes of the wrb-1 deletion mutants tm5532 and tm5938 with those of asna-1(ok938) mutants.

wrb-1(tm5938); svIs135 animals had mCh::SP12-negative foci of GFP::SEC-61β that indicated a TA protein-targeting defect, although this phenotype was less pronounced than that seen in wrb-1(RNAi); svIs135 animals or in asna-1(ok938); svIs135 animals (Figure 14A). Similar to the case in asna-1(ok938) animals, the SP12 marker appeared in a more agglomerated pattern in wrb-1(tm5938) animals than in controls. However, several differences in body morphology between asna-1 and wrb-1 mutants were found. Most obviously, both wrb-1 mutants were darker and bigger than the asna-1(ok938) mutant (Figure 14B). Further, asna-1(ok938) hermaphrodites were sterile with severely underproliferated germlines that were incapable of forming oocytes or sperm (Paper III). Loss of oocyte
production due to underproliferated germlines is an IIS-related phenotype (Lopez et al., 2013). Although both mutants for wrb-1 were also sterile (Figure 14C), their gonads were better developed and had both oocytes and sperm (Paper III). Interestingly, injection RNAi against wrb-1 did not result in complete sterility (Figure 14C), although their TA protein-targeting defects were more pronounced than in wrb-1 mutants.

**Figure 14. WRB-1 is required for tail-anchored protein targeting and fertility**

A. Confocal micrographs of suf135 animals co-expressing GFP::SEC-61β and mCh::SP12. The white arrows indicate GFP::SEC-61β foci that do not co-localize with the mCh::SP12 marker. White squares indicate the area enlarged in the right most panels. Scale bars are 20 µm.

B. Micrographs showing body size comparisons between asna-1(ok938) and wrb-1(tm5938) mutants.

C. Brood size comparisons between mock injected controls, wrb-1(RNAi) animals and wrb-1(tm5938) mutants. Progeny from each mother was scored. Error bars are +/- SEM for 4 control, 8 wrb-1(RNAi)-injected and 8 wrb-1(tm5938) mothers.
In summary, the *wrb-1* mutants had better-developed gonads than *asna-1* mutants. Although both *asna-1* and *wrb-1* mutants were sterile, *wrb-1* mutants were substantially bigger. This demonstrates that the morphological defects between *wrb-1* and *asna-1* mutants only partially overlap. If WRB-1 is completely indispensable for ASNA-1-dependent TA protein targeting, as suggested for its yeast (Wang et al., 2011) homolog, then *asna-1* is likely to have additional functions besides its role in TA-protein targeting. However, the differing phenotypes of *wrb-1* and *asna-1* mutants could be due to greater maternal rescue of *wrb-1* mutants coming from *wrb-1(+/balancer)* animals. However, injection RNAi against *wrb-1* presumably reduced the levels of maternally contributed WRB-1 to less than in *wrb-1(-/-)* animals segregating from heterozygous mothers. But while injection RNAi produced a stronger defect in TA protein targeting, it did not recapitulate the growth and germline defects of *asna-1*.

**Analysis of additional predicted components of the C. elegans tail-anchored protein targeting machinery**

As a member of the pre-targeting complex for TA protein targeting, the yeast Get4 protein is thought to deliver TA proteins to Get3/ASNA-1 (Chang et al., 2010). *C. elegans* CEE-1 shares 27% identical sequence with Get4 and represents the best match for Get4 in the *C. elegans* genome. Another protein that is also part of the pre-targeting complex in yeast is Sgt2, which acts to bind transmembrane domains of TA proteins and hand them over to Get3/ASNA-1 via Get4 and Get5 (Wang et al., 2010). The best *C. elegans* match for Sgt2 is SGT-1, with its 29% identical sequence and its three conserved tetratricopeptide repeat domains. Since both Get4 and Sgt2 are important for TA protein targeting to ER membranes in yeast, their homologs were investigated for TA protein targeting functions in *C. elegans*.

Similar to *asna-1(ok938)* and *wrb-1(tm5938)*, agglomerated ER morphology was observed in *svIs135* animals upon *cee-1(RNAi)* ([Figure 15](#)). However, at this level of inactivation, no non-ER localization of GFP::SEC-61β could be observed although it was clear that *cee-1(RNAi)* was working because the brood size of these animals was around 65% (*n=4* mothers) compared to that of mock injected controls (*n=4* mothers). Upon *sgt-1(RNAi)*, no effect on either ER morphology or GFP::SEC-61β targeting was detected ([Figure 15](#)). Since neither growth, morphology nor fertility was affected in *sgt-1(RNAi)* animals, compared to mock-injected controls, it cannot be ruled out that the RNAi was ineffective. Also, the deletion allele *sgt-1(ok1000)* is available from the CGC stock center. But since we were not able to detect a polymorphism in the ok1000 mutant by genomic PCR it was not used.
**Figure 15. Tail anchored protein targeting analysis in cee-1 and sgt-1-depleted animals**

Confocal micrographs of *cis*Is135 animals subjected to injection RNAi. *cee-1(RNAi)* animals had patches of the ER marker mCh::SP12 that had an agglomerated appearance. In many cases these patches were devoid of GFP::SEC-61β (arrow). Scale bars are 20 μm.

**asna-1 and wrb-1 mutants have ER and Golgi morphology defects and accumulate cytosolic inclusion bodies**

The confocal analysis indicated that the ER morphology was likely altered in *asna-1* and *wrb-1* mutants. Both *asna-1* and *wrb-1* showed agglomerated patterns of mCh::SP12 localization, even though the mCh::SP12 reporter successfully targeted to ER membranes. To investigate the nature of this agglomerated ER morphology, we analysed intestinal cells of wild type and mutant animals with transmission electron microscopy (TEM). Strikingly, the ER lumen was greatly dilated in both *asna-1(ok938)* and *wrb-1(tm5938)* animals (Figure 16A). This luminal dilation was present in all sections of all *asna-1(ok938)* and *wrb-1(tm5938)* animals analysed. We also found ER-containing autophagosomes (ERAs) in both of these mutants, although they were much more common in *wrb-1(tm5938)* (Figure 16A). The formation of ERAs was previously reported to counterbalance ER stress under UPR-inducing conditions in yeast (Bernales et al., 2006), suggesting that the observed ER membrane remodelling in *asna-1(ok938)* and *wrb-1(tm5938)* could be due to elevated ER UPR. Indeed, *asna-1(ok938)* animals displayed a robust induction of an hsp-4::GFP transcriptional reporter (Paper III) and RNAi against *asna-1* has previously been reported to induce the ER UPR.
(Natarajan B., 2013). Since ER UPR transcription factors are known to control expression of several genes regulating autophagy (Bernales et al., 2006; Bernales et al., 2007; Ciechomska et al., 2013; Yorimitsu et al., 2006), we investigated whether autophagy was induced in asna-1(ok938) animals. LGG-1 is the C. elegans homolog of yeast Atg8 and mammalian MAP-LC3. A translational mCherry::LGG-1 reporter is diffusely dispersed in the cytoplasm of intestinal cells, but localizes to autophagosomal foci when autophagy is induced (Gosai et al., 2010). We observed that mCherry::LGG-1 localized more diffusely in wild-type animals and more punctate in asna-1(ok938) animals, suggesting that autophagy was indeed induced in asna-1(ok938) animals (Paper III).

Retrograde transport of proteins from Golgi to ER is defective in yeast GET1/wrb-1 and GET2 mutants (Schuldiner et al., 2005). But no direct evidence has been presented of a role for GET3/asna-1 in Golgi function and no morphological analysis of the Golgi compartment has been presented for GET1/wrb-1 or GET2 mutants. Our TEM analysis showed dramatic effects on Golgi morphology in both asna-1(ok938) and wrb-1(tm5938) animals (Figure 16B). In wrb-1(tm5938) animals we could not find any assembled Golgi stack in any analysed animal, even though Golgi complexes were numerous and easily detected in all analysed wild-type animals. In several places, however, we observed patches of small vesicles that may indicate fragmented Golgi complexes (Pavelka et al., 2010) and (Figure 16B). However, in asna-1(ok938) animals we were able to find Golgi complexes. But strikingly, and in contrast to wild type that always had more or less biconcavely shaped Golgi stacks, all detected Golgi stacks (14/14) in asna-1(ok938) animals were unidirectionally bent into crescent shapes (Figure 16B). In addition, the width of these crescent-shaped Golgi stacks was less than in wild type stacks. Interestingly, crescent-shaped Golgi stack architecture has been reported in situations where secretory trafficking is compromised (Brux et al., 2008; Friggi-Grelin et al., 2006). This is consistent with the previously described DAF-28/insulin secretion defect in asna-1-depleted animals (Kao et al., 2007).

In yeast GET2 mutants and when depleting yeast cells of energy, TA protein insertion into the ER membrane is likely to be compromised. Under these conditions, yeast cells display “deposition sites” that might contain TA proteins (Powis et al., 2013). Strikingly, both asna-1(ok938) and wrb-1(tm5938) mutants displayed numerous proteinacious inclusion bodies in the cytosol of intestinal cells (Figure 16C). In some instances in asna-1(ok938) cells, these inclusion bodies were flanked by small membrane
Figure 16. Overlapping ultrastructural phenotypes of asna-1 and wrb-1

TEM images of intestinal cells in wild type, asna-1(ok938) and wrb-1(tm5938) animals. All images are taken at 80000 x magnification. Scale bars are 0.2 µm.

A. The ER lumen is dilated in intestinal cells from asna-1(ok938) and wrb-1(tm5938) mutants compared to wild type (top panels). Arrowheads indicate Rough ER (RER) membranes. Portions of RER were found to be engulfed in ER-containing autophagosomes (ERAs) in asna-1(ok938) and wrb-1(tm5938) animals (bottom panels). Arrows indicate ERAs.

B. The Golgi stack morphology is biconcave in wild type, but crescent-shaped in asna-1(ok938) and probably disintegrated in wrb-1(tm5938). Arrows indicate wild type and asna-1(ok938) Golgi stacks and a candidate disintegrated Golgi compartment in a wrb-1(tm5938) animal.

C. Examples of the numerous cytosolic inclusion bodies (arrows) found in intestinal cells of asna-1(ok938) and wrb-1(tm5938) animals. The arrowheads point to examples of membrane whorls that in some cases flanked the inclusion bodies in intestinal cells of asna-1(ok938) animals.
whorls, but were otherwise devoid of membranes in both asna-1(ok938) and wrb-1(tm5938) cells. The inclusion bodies were of variable sizes, ranging from about 0.2 to 0.5 µm in diameter in asna-1(ok938), and slightly smaller in wrb-1(tm5938). We note that in terms of size, these inclusion bodies roughly correspond to the non-ER GFP::SEC61β foci seen in the confocal analysis of asna-1(ok938) and wrb-1(tm5938) mutants. A tempting hypothesis is that TA proteins that fail to incorporate into the ER membrane instead accumulate and aggregate in the cytosol due to the hydrophobic nature of their transmembrane domains.

SEC-61β is both a TA protein that requires ASNA-1 and WRB-1 for correct ER targeting (Figure 13A and 14A) and a predicted component of the SEC-61 translocon, which is required for co-translational, signal recognition particle (SRP)-dependent protein targeting to the ER. All of the observed phenotypes in asna-1(ok938) and wrb-1(tm5938) animals could therefore hypothetically have been caused by defective protein targeting in the SRP pathway. However, both Golgi and ER morphology was normal in sec-61β(tm1986) mutants (Paper III). In addition, no signs of proteinacious inclusion bodies were detected in any of the analysed sec-61β(tm1986) animals. These observations suggest that the inclusion bodies and the organellar defects seen in asna-1(ok938) and wrb-1(tm5938) mutants were not likely due to defective SEC-61-dependent, non-TA protein targeting. The defects rather appear to correlate with defects in ASNA-1 and WRB-1-dependent TA protein targeting.
DISCUSSION

Identification of IIS and DAF-28 secretion regulators

While most regulators of IIS and insulin secretion in C. elegans have been identified in screens for longevity or dauer formation defects, we instead performed an RNAi screen for asna-1-like growth defects using a set of genes representing a predicted ASNA-1 interactome. This approach was successful in identifying two new regulators of IIS and DAF-28/insulin secretion. More regulators of IIS and DAF-28 secretion could likely be identified in a larger screen using slow growth in spite of normal feeding as criteria. Also, the results presented here suggest that the likelihood of finding new IIS regulators in such screens can be enhanced if they are performed in predicted interactomes of known IIS regulators. In this manner, the efficiency of such screens may be boosted by previous large scale systems biology studies and genome-wide yeast two-hybrid screens that have identified and predicted interactomes using data from various organisms.

Although screening through the predicted ASNA-1 interactome was successful in the identification of new IIS regulators, it is important to recognize that ASNA-1 homologs in various organisms have other, possibly IIS-unrelated functions. They promote both metal resistance (Hemmingsson et al., 2009b; Shen et al., 2003; Tseng et al., 2007) and targeting of tail-anchored proteins to the ER (Mateja et al., 2009; Schuldiner et al., 2008; Stefanovic and Hegde, 2007). In yeast, Get3 also has a role in G-protein signalling (Lee and Dohlman, 2008), can bind to a non-TA chloride channel (Metz et al., 2006) and has a role in the ubiquitin-proteasome system (Auld et al., 2006). Therefore some of the predicted interactors could be involved in other ASNA-1-related functions than IIS and insulin secretion and thus fail detection in a screen based on the IIS phenotype. This notion is supported by previous work in our lab showing that the IIS promoting activity of asna-1 can be genetically separated from its involvement in metal resistance (Hemmingsson et al., 2010). Therefore, the predicted interactome could be screened for defects in other asna-1-dependent functions and thereby perhaps identify genes required for cisplatin resistance and TA protein targeting. Importantly, one of the IIS regulators identified from the predicted ASNA-1 interactive, ENPL-1 [which is described elsewhere: (Natarajan, 2012)], was shown to physically interact with ASNA-1. Therefore it is reasonable to assume that screening through the predicted interactome for other asna-1 phenotypes could lead to the identification of physical interactors required for other aspects of ASNA-1 function. However, in this thesis the set of genes was used solely to find new regulators of IIS. In
doing so, ykt-6 and tomm-40 were successfully identified as positive regulators of growth, IIS and DAF-28/insulin secretion. Both of these genes have previously, like asna-1 itself, not been identified as IIS regulators in screens for longevity or dauer development defects, likely because RNAi against ykt-6 or tomm-40 does not cause dramatic effects on longevity or dauer formation. Depletion of either one of these genes instead causes larval arrests to a large extent before the dauer stage is reached.

Conversely, the screen was also successful in removing two candidate IIS regulating genes, rps-0 and iars-1, from further studies because the growth defects associated with RNAi against them were not suppressed in daf-16 mutants and were therefore not likely due to reduced IIS pathway activity.

**Functional mitochondria promote DAF-28 secretion and IIS-dependent growth**

In TOMM-40-depleted animals, several aspects of mitochondrial function were affected. First, the ability to import a mitochondrial protein was compromised. Second, the mitochondrial unfolded protein response (UPR) was triggered. And third, the ability to produce an electrochemical potential across the inner mitochondrial membrane was reduced. These effects demonstrate a crucial role for TOMM-40 in mitochondrial function. In yeast, virtually all nucleus-encoded proteins destined for the mitochondria must first pass the outer mitochondrial membrane through the protein-conducting pore presented by Tom40 [reviewed in (Mossmann et al., 2012)]. Since most mitochondrial proteins are encoded in the nucleus and synthesized in the cytosol, protein import through TOMM-40 is likely an upstream determinant of mitochondrial function. In that way, reducing levels of TOMM-40 may reduce mitochondrial function in a broad and non-specific manner, presenting an opportunity to study various processes in a background that is bluntly sensitized for mitochondrial function.

When mitochondrial function is compromised by inhibition of mitochondrial DNA replication or translation, worms can arrest growth in the L3 stage (Tsang and Lemire, 2002; Tsang et al., 2001). This is thought to be due to the failure in these animals to meet a steeply increased energy requirement at the L3/L4 molt. tomm-40(RNAi)-treated animals rarely grew beyond the L3 stage and had defective mitochondria. However, the growth defect was suppressed in daf-16 mutants, so that daf-16(mgDf50); tomm-40(RNAi) animals were more capable of growing beyond the L3/L4 molt. This is suggestive of a daf-16 component to the growth arrest before the L3/L4 molt.
A set of other genes, belonging to the mitochondrial ribosomal protein class, was also found to affect growth in a \textit{daf-16}-dependent manner and to cause upregulation of the mitochondrial unfolded protein response (mtUPR) (Billing et al., 2012; Natarajan, 2012). A second study showed that inactivation of genes in this class created a stoichiometric imbalance in mitochondria between nucleus-encoded and mitochondria-encoded proteins, which evoked the mtUPR (Houtkooper et al., 2013). It is possible that the mtUPR activation upon \textit{tomm-40}(RNAi) is also due to stoichiometric imbalances, since protein import of nucleus-encoded proteins appeared diminished. Also, while increases in the production of reactive oxygen species (ROS) in mitochondria can evoke the mtUPR (Yoneda et al., 2004), no signs of increased ROS upon \textit{tomm-40}(RNAi) were detected. However, a distorted imbalance between the load of unfolded proteins and the folding capacity within mitochondria cannot be ruled out as a possible cause of the mtUPR activation.

Given the central role for mitochondria in ATP synthesis, fat metabolism, and Ca\textsuperscript{2+} signalling, the broadly dysfunctional mitochondria generated by \textit{tomm-40}(RNAi) could inflict a wide span of defects that could affect growth in several ways. However, suppression of the growth defect by \textit{daf-16}, nuclear localization of DAF-16::GFP and defective secretion of DAF-28 strongly suggests that IIS was weakened upon TOMM-40-depletion and that the weakened IIS may have contributed to the growth defect. In addition, the nuclear localization of DAF-16::GFP seen upon \textit{tomm-40}(RNAi) was reversed by over expressing DAF-28, suggesting that defective DAF-28 secretion is a contributing factor of the reduced IIS in \textit{tomm-40}(RNAi) animals. This notion was further supported by the apparent specificity in the secretion defect: secretion of DAF-28 was inhibited, while secretion of two other dense-core vesicle-dependent neuropeptides, INS-22 and ANF, was normal. Together, these lines of evidence suggest a model whereby signals of nutrient availability are conveyed through functional mitochondria to promote DAF-28 secretion and downstream IIS-dependent growth.

In addition, the DAF-28/insulin secretion defect was pheonocopied by RNAi against other mitochondrial genes. RNAi against \textit{tomm-20} and \textit{tomm-22}, and against genes encoding mitochondrial ribosomal proteins all resulted in defective DAF-28::GFP secretion. \textit{mev-1(kn1)} mutants also secreted less DAF-28::GFP. Interestingly, RNAi against \textit{tomm-20} and \textit{tomm-22} did not affect secretion of INS-22, suggesting that the discrimination between secretion mechanisms among different \textit{C. elegans} ILPs can indeed be made based on their dependence on active mitochondria. However, while humans have separate receptors for insulin, IGFs and relaxins, \textit{C. elegans} only has a single insulin/IGF receptor, DAF-2. But signalling through DAF-2 does not
necessarily activate the AGE-1/AKT-1/DAF-16 cascade, and can for instance instead activate the RAS-ERK signalling pathway (Lopez et al., 2013). Hence, DAF-2 may perhaps direct signalling outputs to different pathways depending on type of ligand activation and may that way provide specificity to signalling outputs for different ILPs.

The transient entry into a semi-dauer stage upon tomm-40(RNAi) and the synergistic effects of dauer formation with daf-7 but not daf-2 also argues that IIS was reduced. The transient nature of entry into the semi-dauer stage suggests that dauer entry rather than dauer escape was affected. This is consistent with the DAF-28 secretion defect, since DAF-28 prevents dauer entry rather than dauer escape (Cornils et al., 2011). Interestingly, this transient entry into a semi-dauer stage resembles a phenotype seen in daf-9 mutants. DAF-9 acts mainly in the endocrine XXX cells to promote reproductive growth downstream of DAF-16, through synthesis of the steroid hormone dafachronic acid (DA) [reviewed in (Fielenbach and Antebi, 2008)]. The central role of mitochondria in fat metabolism and the clear appearance of TOMM-40-depleted animals could indicate defective fat metabolism. Since DA synthesis depends on functional fat metabolism (Butcher et al., 2009), the possibly that TOMM-40-depletion also affects DA synthesis with concomitant effects on reproductive growth cannot be ruled out.

**Defective Golgi precludes DAF-28/insulin secretion**

The vesicle-SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor) protein YKT-6 is highly conserved from yeast to humans. Unlike other SNARE proteins it lacks a membrane anchor but can instead prenylate itself at the C-terminus to promote its membrane association (Veit, 2004). As the *C. elegans* homolog YKT-6 also lacks a transmembrane domain but instead contain a robust prenylation motif in its C-terminus, it is likely that it too can associate with membranes in a similar fashion [prediction softwares: (Kall et al., 2004; Maurer-Stroh and Eisenhaber, 2005)]. In yeast, Ykt6 was first implicated in the secretory pathway at an early Golgi stage (McNew et al., 1997). Later research showed that it also participates in several vesicle-to-vacuole targeting events and that it is required for macroautophagy (Nair et al., 2011). In humans, YKT6 is highly enriched in the brain, where it localizes to, as of yet, unidentified cytosolic structures (Hasegawa et al., 2003). In other cells it appears to act in ER to Golgi transport as its yeast homolog (Xu et al., 2002). *C. elegans* YKT-6 has previously been reported to promote growth and the proper localization of a Golgi-resident protein (Maekawa et al., 2009). Here, we confirm a growth defect upon *ykt-6(RNAi)* and show that YKT-6 is a positive regulator of IIS.
In addition, *ykt-6*(RNAi) animals could produce, but not secrete DAF-28::GFP properly. Instead DAF-28::GFP accumulated with a granular appearance, suggestive of a blocked secretion. Similarly, in *C. elegans* intestinal cells, depletion of the Golgi residing transport protein SNAP-29 causes vesiculation of the Golgi compartment, inhibition of yolk protein secretion and accumulation of small cargo vesicles (Sato et al., 2011). Homologs of both YKT-6 and SNAP-29 are required in mammalian cells for constitutive secretion (Gordon et al., 2010), where siRNA against Ykt6 causes entrapment of signal sequence (SS)::GFP protein in the Golgi compartment.

It is known that the yeast Get3 receptor subunit Get2 is indispensible for ER to Golgi transport (Schuldiner et al., 2005). It is also known that defects in retrograde Golgi to ER transport precedes Golgi defects, ER stress and protein aggregation in a cell line model for amyotrophic lateral sclerosis (Atkin et al., 2013). As asna-1(ok939) animals display Golgi morphology defects, ER stress and protein aggregation, it is possible that they also have defects in trafficking between the ER and Golgi compartments. This represents a possible connection to the Golgi-resident transporter YKT6 that, similar to ASNA-1, is implicated in both Golgi function and DAF-28 secretion. Interestingly, the function of YKT-6 in Golgi transport requires the TA protein GS28 in *C. elegans* (Maekawa et al., 2009). Therefore the insulin secretion defect of ASNA-1-depleted animals could possibly be due to a TA protein-dependent Golgi defect. In support of this hypothesis, both asna-1(ok938) and wrb-1(tm5938) animals were defective in TA protein targeting and had defective Golgi morphology. However, the Golgi morphology defects in asna-1(ok938) and wrb-1(tm5938) animals appeared very different, and may not have had the same causes or consequences.

**DAF-28 secretion is mechanistically similar to mammalian insulin secretion**

The insulin-like peptide (ILP) DAF-28 has several hallmarks of a metabolically regulated insulin. In the IIS pathway, DAF-28 acts upstream and agonistically to the receptor daf-2 in preventing entry into the dauer stage when food is available (Cornils et al., 2011; Li et al., 2003). Like human insulin its expression levels are sensitive to nutrient availability. DAF-28 levels are high when nutrient levels are high and low when nutrient levels are low (Li et al., 2003). Another level of similarity between mammalian insulin and DAF-28 is at the level of secretion. As insulin secretion from pancreatic islets is enhanced upon blockage of rectifying K+ currents (Herrington et al., 2006), so is DAF-28 secretion enhanced by a mutation in the rectifying K+ channel exp-2 (Park et al., 2012). In addition, secretion of both mammalian
insulin and *C. elegans* DAF-28 requires ASNA-1 (Kao et al., 2007) as well as CAPS/UNC-31 (Speese et al., 2007), the latter demonstrating their dependence on the dense core vesicle (DCV) machinery. Since DCVs are formed through budding off from the trans-Golgi network, it is possible that impaired Golgi function in *ykt-6(RNAi)* animals interfered with DCV formation. Indeed, the granular appearance of DAF-28::GFP in *ykt-6(RNAi)* animals suggested that DAF-28::GFP was trapped in some membrane-enclosed compartments that might correspond to Golgi. In any case, the requirement of functional Golgi for DAF-28 secretion supports the notion that the secretory machineries of insulin and DAF-28 are mechanistically similar.

Humans have ten identified members of the insulin-like peptide (ILP)/relaxin superfamily (Claeys et al., 2002). Among these, the role of insulin in metabolism and the roles of IGF1 and IGFII in growth, cell proliferation and lifespan have been extensively studied (Nakae et al., 2001). Some of the relaxin members are known to regulate reproductive processes, but the functions of other members are less established (Sherwood, 2004). As *C. elegans* has 40 ILPs, it remains unclear which of these that functionally resemble IGFs, controlling growth and cell proliferation, and which that resemble insulin, controlling metabolism in response to nutritional signals. In mammals, dependence on the level of mitochondrial activity is a characteristic of insulin secretion and not of IGF secretion.

By this categorization, DAF-28 resembles an insulin rather than an IGF, since its secretion depends on mitochondrial activity. Diminishing mitochondrial function by *tomm-40(peRNAi)* did not affect synthesis but rather secretion of DAF-28. The secretion defect appeared somewhat specific to DAF-28, since two other neuropeptides were secreted normally. Interestingly, one of the normally secreted neuropeptides was the ILP INS-22, suggesting that a distinction can be made among the many *C. elegans* ILPs in their dependence on functional mitochondria for secretion. In addition, further evidence for a mitochondrial involvement in DAF-28 secretion was observed in *mev-1(kn1)* mutants. These mutants are short lived, have increased levels of reactive oxygen species (ROS) and are hypersensitive to further increases of oxidative stress. Although they are defective in complex III of the mitochondrial respiratory chain, they have normal levels of ATP. This is most likely due to a compensatory increase in glycolytic ATP generation, as indicated by their high lactate levels (Senoo-Matsuda et al., 2001). Secretion of DAF-28 was low in *mev-1(kn1)* mutants. But this could be for several reasons because of the pleiotropic phenotypes of these animals. However, the secretion defect observed is consistent with the
previously reported nuclear accumulation of DAF-16::GFP in *mev-1(kn1)* animals (Kondo et al., 2005).

Treatment with high, but sub-lethal levels of the unspecific ROS inducer methyl viologen also caused DAF-28::GFP secretion defects. This secretion defect together with entry into a semi-dauer stage in some animals suggested that IIS might have been reduced upon methyl viologen treatment. Importantly, as mammalian insulin secretion is sensitive to high levels of ROS, so is apparently also secretion of DAF-28. On the other hand, work done in rat islets of Langerhans proposed that low levels of mitochondrial ROS could instead serve as a nutritional signal to stimulate insulin secretion (Leloup et al., 2009). We tested this in worms with low levels of rotenone and found that DAF-28::GFP secretion was enhanced. Hence, DAF-28 seems to be under control of similar signals in coupling the metabolic activity of mitochondria to secretion, as is mammalian insulin. These lines of evidence also support the notion that there are dual effects of ROS in the modulation of insulin secretion. High levels are inhibitory and low levels are stimulatory, which implies that fine-tuning of ROS by quenchers, such as catalases and dismutases, should play important roles in controlling insulin secretion. This can now be assessed using *C. elegans* genetics, in a way that is likely to be relevant to mammals since secretion of DAF-28 in many ways resembles secretion of mammalian insulin.

In conclusion, similar to mammalian insulin, the secretion of DAF-28 is sensitive to various mitochondrial dysfunctions, to Golgi dysfunctions and to high levels of ROS, but is promoted by functional mitochondria and low levels of mitochondrial ROS.

**ASNA-1 and WRB-1 promote tail-anchored protein targeting**

The lack of experimental data from *in vivo* metazoan systems has constrained the analysis of TA protein targeting processes and how they relate to other functions in multicellular organisms. Presented here is the first model for TA protein targeting in live animals.

In *C. elegans*, the ATPase ASNA-1 and its ER-residing, putative receptor WRB-1 were both required for correct targeting of the TA protein SEC-61β to the ER membrane. In the absence of ASNA-1 or WRB-1, SEC-61β accumulated in foci that were not part of the ER membrane. Conversely, a homolog of the TA protein cytochrome b5 that was previously described to target to the ER membrane independently of ASNA-1 (Favaloro et al., 2008) was confirmed to do so also in the *C. elegans* model. This suggests that the fidelity of this model allows a discrimination to be made among TA proteins.
in their relative dependence on ASNA-1 for correct targeting to the ER membrane. In addition, non-ER localization in foci, which was used as a criteria for defective TA protein targeting, is consistent with previous findings in yeast Get3/ASNA-1 and Get1/WRB-1 mutants, where TA protein proteins were aggregating in cytosolic foci or misinserted into mitochondria (Powis et al., 2013; Schuldiner et al., 2008). The resemblance with yeast mutant phenotypes, and the structural conservation of ASNA-1 and WRB-1 proteins from yeast to humans, supports the notion that results generated from the C. elegans TA protein targeting model are likely valid in other organisms as well.

Surprisingly, RNAi against predicted components of the pre-targeting complex, cee-1/GET4 and sgt-1/SGT2, did not produce any detectable TA protein targeting defects. While RNAi against sgt-1 may have been ineffective, RNAi against cee-1 caused both a decreased brood size and an agglomerated ER morphology. Both of these phenotypes were reminiscent of RNAi against wrb-1. But the normal TA protein targeting in cee-1(RNAi) animals argues against a crucial role in that process. However, the interpretation of RNAi results calls for caution as the effectiveness of RNAi can vary for different genes and between experiments. Indeed, both the brood size phenotype and the ER morphology phenotype in cee-1(RNAi) animals were similar but milder than in wrb-1(RNAi) animals. It is therefore possible that the RNAi effectiveness was less for cee-1 than for wrb-1 and that a stronger cee-1-inactivation could recapitulate the full spectrum of wrb-1 phenotypes.

The confocal- and transmission electron microscopy analysis showed that both asna-1(ok938) and wrb-1(tm5938) mutants had a swollen ER lumen. The swelling was accompanied by a sequestration of ER into ER-containing autophagosomes (ERAs), which together with the high ER stress and increased autophagy in asna-1(ok938) mutants indicated a stress-induced remodelling of the ER membrane in these animals. However, as observed by confocal microscopy, asna-1(ok938), wrb-1(tm5938) and cee-1(RNAi) animals all had some patches of the ER that appeared unavailable for insertion of TA proteins. These patches could supposedly correspond to the observed ERAs and other compartments involved in macroautophagy, such as autolysosomes. In support of this, ER patches devoid of SEC-61β were also seen upon starvation (Paper III), a condition that is known to induce autophagy. The ER patches were also observed upon DTT treatment (Paper III), which in yeast is known to induce ER stress, dilation of the ER lumen and sequestration of the ER into ERAs (Bernales et al., 2006). But importantly, neither starvation nor DTT treatment resulted in defective TA protein targeting. Therefore the swollen ER patches that are devoid of TA
proteins in \textit{asna-1} and \textit{wrb-1} mutants are not likely correlated to TA protein targeting defects.

Another explanation for the ER patches devoid of TA protein markers could be that SP12 is able to enter portions of the ER, from which the TA protein markers are retained. Indeed, selective retention mechanisms do exist between rough ER and smooth ER membranes, and in \textit{C. elegans} the SP12 marker is known to be present in both (Rolls et al., 2002). It is therefore possible that both SEC-61\(\beta\) and CYTB5.1 are restricted to the rough ER whereas SP12 is present in both rough and smooth ER. This notion was somewhat supported by the observation that even some otherwise wild-type animals had small patches of SP12 that were not decorated by the SEC-61\(\beta\) marker (\textbf{Figure 14A}). In addition, it has been suggested that \textit{C. elegans} intestinal cells contain very little, if any, smooth ER (Rolls et al., 2002). Therefore the SP12 patches devoid of TA protein markers in otherwise wild-type animals could perhaps indicate the presence of a small portion of smooth ER in intestinal cells.

\textit{wrb-1\textit{(RNAi)}}-treated animals had more pronounced TA protein targeting defects compared to \textit{wrb-1\textit{(tm5938)}} mutants. This indicates a strong maternal effect for WRB-1 in its TA protein targeting activity. But conversely, \textit{wrb-1\textit{(tm5938)}} mutants had more pronounced fertility defects compared to RNAi-treated animals, which were always capable of producing few, but viable offspring. Therefore the levels of functional WRB-1 protein later in life could perhaps be higher upon RNAi, and be more important for fertility, and the levels of WRB-1 early in life could be higher in mutants, and be more important for the TA protein targeting capacity of intestinal cells. This would suggest a difference in the temporal requirement for WRB-1 in gonads and in intestinal cells. Indeed, all intestinal cells are born during embryogenesis, while both embryonic and post-embryonic cell divisions occur during gonadogenesis. A requirement for WRB-1 during mitosis could therefore perhaps explain the opposing strengths in fertility- and TA protein targeting defects in mutants vs. RNAi-treated animals. However, it is also possible that \textit{wrb-1\textit{(RNAi)}} generated stronger TA protein targeting defects and weaker fertility defects simply because the RNAi was more effective in intestinal cells than in gonads.

In yeast \textit{Get2} mutants, it was demonstrated that Get3/ASNA-1 moved to proteinacious aggregates in the cytosol (Powis et al., 2013). It was speculated that these aggregates contained TA proteins that had failed to be inserted into the ER membrane. Consistent with these findings in yeast, proteinacious aggregates were observed in both \textit{asna-1\textit{(ok938)}} and \textit{wrb-1\textit{(tm5938)}} animals. It is possible that uninserted TA proteins formed these
aggregates, which were similar in size to the non-ER foci of GFP::SEC-61β observed in the confocal analysis.

Lastly, the palette of ultrastructural phenotypes associated with *asna-1(ok938)*, was largely overlapping with those seen in in *wrb-1(tm5938)*. But none of these phenotypes were seen in *sec-61β(tm1986)* animals, indicating that they are not likely due to defective SEC-61-dependent protein targeting. Instead they appear to correlate with defects in ASNA-1 and WRB-1-dependent TA protein targeting.

**asna-1 is likely to have wrb-1-independent functions**

WRB-1 shows structural homology to human WRB and has a conserved three-transmembrane domain topology and a weakly predicted coiled-coil domain between the first two transmembrane domains. The coiled-coil domain in Get1 and is important for stabilizing the open configuration of Get3 (Mariappan et al., 2011), thereby promoting substrate release at the ER membrane. Importantly, the lack of Get1 in yeast completely abolishes Get3 association with the ER membrane (Auld et al., 2006) and Get3-mediated TA protein targeting to the ER *in vitro* (Wang et al., 2011). But if the human components of the ASNA1-TA protein receptor, WRB and CAML, are co-expressed in yeast Get1/Get2 double mutants, Get3-mediated TA protein targeting to the ER is restored (Vilardi et al., 2014). Therefore WRB-1 homologs appear required for ASNA-1-dependent TA protein targeting and are functionally well conserved.

In different ASNA-1-deplete conditions, injection RNAi against *asna-1* produces the strongest growth defect with an L1 arrest phenotype (Kao et al., 2007). Mutants for ASNA-1 instead have a weaker phenotype of slow growth, probably due to maternal contribution of ASNA-1. However, the L1 arrest phenotype seen upon injection RNAi against *asna-1* is reversible, and as the RNAi effect eventually wears off, worms grow up to become fertile adults. This implies that presence of ASNA-1 during embryonic development is important for growth and to bypass the L1 arrest, which is considered an affect of weakened IIS in these animals (Kao et al., 2007). However, the same growth dependence is not seen during embryonic development for WRB-1, as injection RNAi against WRB-1 does not hamper larval growth. Instead, these injected worms successfully execute all larval molts and can even produce some viable offspring as adults, even though the TA targeting defects were severe in these animals.

Although the differences in growth between *asna-1(RNAi)* animals and *wrb-1(RNAi)* animals could arise from differences in the strength of RNAi
inactivation, they are still suggestive of different requirements for ASNA-1 and WRB-1 for growth. Indeed, also when comparing asna-1(ok938) to two different urb-1 mutants, the urb-1 mutants grew better and had better developed gonads. Since the growth defects of asna-1(ok938) mutants are likely due to reduced IIS (Kao et al., 2007), WRB-1 may be dispensable for IIS. Because Get1 appears absolutely required for TA protein targeting in yeast, and because WRB-1 is also required for TA protein targeting in C. elegans, TA protein targeting could perhaps be dispensable for growth and IIS. Another possibility could be an opposite relation, such that reduced IIS causes TA protein targeting defects. But this did not appear to be the case, since starved animals, which have reduced IIS, did not have defective TA protein targeting (Paper III). In addition, since both asna-1(ok938) and urb-1(tm5938) animals had swollen ER, which is a sign of ER stress, we induced ER stress to similar levels as in asna-1(ok938) using DTT. But this too did not affect TA protein targeting.

The notion that ASNA-1 promotes IIS independently of its role in TA protein targeting is supported by an experiment where a dimerization-deficient version of ASNA-1, likely lacking the ability to promote TA protein targeting, was able to rescue growth (Hemmingsson et al., 2010). This experiment also demonstrated that the same dimerization-deficient version of ASNA-1 was not able to suppress the sensitivity to cisplatin exposure, which indicates that cisplatin resistance is mediated through a TA protein-dependent mechanism. urb-1(tm5938) mutants were also sensitive to cisplatin (result not shown), strengthening the hypothesis that asna-1 promotes cisplatin resistance through a urb-1 and TA protein-dependent mechanism and IIS through a TA protein-independent mechanism. However, a detailed analysis of the IIS pathway in urb-1-depleted animals and in animals expressing dimerization-deficient ASNA-1 will be required to adequately test that hypothesis.

In conclusion, while ASNA-1 and WRB-1 are both required for TA protein targeting to the ER, they may have separable roles in terms of growth and IIS. This challenges the widespread assumption that all the pleiotropic defects associated with asna-1 are consequences of defective TA protein targeting.
FUTURE DIRECTIONS

A screen for new insulin secretagogues

The prevalence of type 2 diabetes has increased dramatically during the last decades and is predicted to go up from 2.8% (171 million) in year 2000 to 4.4% (366 million) in year 2030 (Wild et al., 2004). Given the serious complications associated with type 2 diabetes, it is therefore becoming an increasing burden to global health. In the early development of type 2 diabetes, inadequate insulin secretion is one of the first occurring events (Wajchenberg, 2007). Treatment with insulin secretagogues represents a major asset to control blood glucose levels and to diminish secondary complications (Fonseca, 2003). However, presently available secretagogues are associated with several adverse effects and eventual deterioration of the therapeutic efficiency [reviewed in (Ahren, 2009)]. There is therefore a great need to find new insulin secretagogues that can sustain long-term treatment efficiency without adverse effects. Work presented in this thesis has identified DAF-28 as a metabolically regulated insulin that is sensitive to rotenone, a known secretagogue for human insulin. Rotenone increased DAF-28::GFP secretion even from wild type levels in larvae, such that DAF-28::GFP was readily seen in their coelomocytes. This suggests that the larval DAF-28::GFP secretion assay could be utilized in a screen to find novel insulin secretagogues. Such a screen could be performed by growing DAF-28::GFP-expressing larvae in the presence of chemicals from chemical compound libraries. Worms that secrete more DAF-28::GFP when grown in the presence of a given compound could be identified using a worm sorter, which can identify the presence of fluorophores in different parts of the body. This approach could provide a high throughput scale in the identification of new candidate insulin secretagogues. Subsequent characterization and analysis of chemical modifications of candidate compounds could then be performed in worm, cell line and pseudo-islet models before being tested in vertebrates.

Characterization of C. elegans insulin-like peptides

Since the secretion of DAF-28 but not INS-22 was sensitive to mitochondrial dysfunction, worm ILPs could likely be grouped based on their dependence on mitochondrial activity for secretion. Such a distinction could be accomplished by employing the same strategy as presented in this thesis for the investigation of DAF-28. By 1) tagging known worm ILPs with GFP and expressing them under their endogenous promoters and by 2) screening through lines expressing these GFP::ILP and identify those that are secreted
to a lesser extent in the tomm-40(peRNAi) background. As a second criterion in such a screen, the effect of rotenone could be utilized to investigate whether an identified mitochondria-dependent ILP is sensitive to a known secretagogue for mammalian insulin secretion or not. Such a screen could indicate which worm ILPs are likely to function more like insulins and which are likely to function more like IGFs. This would add to the current knowledge of C. elegans ILPs and their regulation of metabolism, cell proliferation and lifespan.

**Benefits and validation of a metazoan model for tail-anchored protein targeting**

The TA protein targeting model set up in this thesis will be advantageous compared to previous biochemical and unicellular models as it can be combined with analysis of processes that are only possible in animals. And the diverse phenotypes of asna-1 animals cannot be fully understood if it is not known how they relate to TA protein biogenesis. Setting up a model for TA protein targeting in C. elegans was a first step to gain such knowledge. However, this model needs better characterization, and especially in terms of genetic- and possible physical interactions between ASNA-1 and WRB-1. First, possible physical interactions need to be elucidated. This can be accomplished by pull-down experiments with GST-tagged ASNA-1 and FLAG-tagged WRB-1. Second, expression of the coiled-coil region of human WRB was shown to interfere in a dominant-negative manner with ASNA1-mediated TA protein insertion (Vilardi et al., 2011). Such an experiment, which could be easily performed in our model, would indicate if the predicted coiled-coil domain in WRB-1 is implicated in ASNA-1 interactions. Third, as initial results point out the promotion of IIS as a possibly TA protein-independent function of ASNA-1, the role of WRB-1 in IIS needs to be analysed. This can be accomplished using the same array of tests for IIS phenotypes that have been described in this thesis. If wrb-1(RNAi) turns out to affect IIS, a screen among known TA proteins in C. elegans for those that affect secretion of DAF-28::GFP would be motivated. Since the entire set of predicted TA proteins in C. elegans is 428 (result not shown), RNAi against these proteins in the svIs69 (daf-28::gfp) strain could identify those that are likely mediating the insulin secretion defect of asna-1 knockdown animals.

On the other hand, if the insulin secretion defect of asna-1 turned out to be a WRB-1-independent function, another route of investigation could be taken. Human ASNA1 has been shown to promote secretion of short, signal sequence-containing peptides in a process that involves the SEC61 translocon (Johnson et al., 2012). If DAF-28 were targeted to the ER in such a process, it could explain the insulin secretion defect of asna-1. Such an
investigation could be performed with a beta-opsin-tagged version of DAF-28. The beta-opsin tag becomes glycosylated once it enters the ER (Abell et al., 2007), which can be detected by a size shift on a western blot. If glycosylated DAF-28 is not detected in asna-1(ok938) lysates it is a strong indication that DAF-28 fail to be targeted to the ER.

To investigate the nature of the TA protein foci observed in the model for TA protein targeting, at least two approaches could be taken. First, using immuno-EM techniques, GFP and mCherry localization could be analysed in svIs135 (gfp::sec-61β; mCh::SP12) animals in both asna-1(ok938) and wrb-1(RNAi) backgrounds. Structures where GFP::SEC-61β localizes without mCh::SP12 would likely correspond to the foci. However, such a method relies on antibody binding to epitopes that may be hidden or buried in an aggregate. Therefore this method may prove to have limited utility. Instead, another method can be used that takes advantage of both fluorescent and electron microscopy techniques. In correlative light and electron microscopy (CLEM), specimens are embedded in a special plastic that preserves the excitability of fluorophores. Sectioned svIs135 animals with asna-1(ok938) or wrb-1(RNAi) backgrounds would then first be imaged by fluorescence microscopy. A structure positive for GFP but negative for mCherry would be labelled in its vicinity with a laser mark. The same grid would then be analysed with transmission electron microscopy to identify the nature of the structure labelled with the laser mark.

In addition, RNAi against cee-1 did not produce a TA protein targeting defect and RNAi against sgt-1 may have been ineffective. CRISPR/Cas9 mutagenesis could instead be used to generate mutant alleles for cee-1 and sgt-1. Investigation of their TA protein targeting function under mutant conditions could probably generate more meaningful results.

These sets of experiments, together with continued investigation of other aspects of TA protein-dependent vs. independent functions of ASNA-1, will provide insight into the biological relevance of TA protein targeting. Because the processes governed by ASNA-1 are of clinical interest in diabetes and cancer treatment, such knowledge may reveal new targets for drug treatment of these diseases.
CONCLUSIONS

1. YKT-6 and TOMM-40 are positive regulators of IIS and DAF-28/insulin secretion.

2. TOMM-40 is a mitochondrial protein translocase that is indispensable for mitochondrial function.

3. Signals of nutrient availability are conveyed through mitochondria to promote DAF-28 secretion and downstream IIS-dependent growth.

4. ASNA-1 and WRB-1 promote targeting of the TA protein SEC-61β to the ER in live *C. elegans* animals.

5. *asna-1* and *wrb-1* mutants have ER and Golgi morphology defects, and accumulate proteinaceous inclusion bodies in the cytosol.

ACKNOWLEDGEMENTS

I was recently told that the word kappa is not only a commonly used term in academia, but is also used for the fat layer that covers some pieces of meat. If kept on during cooking, it can give flavour and juiciness to the meat. I am a vegetarian since 15 years and feel a bit awkward about this metaphor, but thank you for holding this piece of fat in your hands. More in particular, I would like to acknowledge the following people that were of particular importance in getting this work done:

I would like to express my deepest appreciation to Gautama Kao, my primary supervisor, scientific tutor and friend. It has been a true pleasure to get to know you and to work with you over the years. Thank you for being such a fantastic supervisor during this time. The amount of help and support you have given me throughout this project is tremendous and this thesis would not have materialized without your help. I will truly miss the good times we had in the lab and elsewhere. You are one of the sharpest scientists and one of the funniest persons I ever met. Who’s bad, if you know what I mean? Peter Naredi, my assistant supervisor and PI, I cannot thank you enough for giving me the opportunity to work in the ASNA-1 project. I will never forget the day when you introduced me to the C. elegans people and put me in this great environment. Also, you were always extremely helpful in everything from commenting on presentations to putting me under the knife (literally). I am very grateful for everything you have done for me, for my project and for this thesis. I am also very grateful to Simon Tuck, my assistant supervisor, for all your advice, for sharing your lab and for hosting all the lab gatherings.

I cannot express my gratitude enough to Lars Nilsson, my dear colleague and one of my best friends. Our daily chats about everything from high to low have made day so many times. Your views and perspectives on science and life have orienteered their way into making daily life in the lab such a delight. Also, thank you for your great suggestions during both paper and thesis writing. Sri Bale Natarajan Ji, my dear colleague and brother in arms, I always appreciated our collaborations and discussions as well as your sharp mind and sense of humour. I am very grateful to Rahul Gaur and Oscar Hemmingsson, for their invaluable scientific and social inputs into the ASNA-1 project. I am also grateful to Ming Sheng and Ágnes Regós for all suggestions and for being such great colleagues. In addition, I would like to acknowledge Ateequrrahman Mohammed, Lenore Johansson,
Eva Johansson, Agneta Rönnlund and Eva-Maj Hägglöv for fantastic technical support. Christina and Anette, thank you for helping me out a lot during the UKBF time and for always taking interest in my project. Viktor Nilsson-Örtman, I am very grateful for all your help with statistics in paper I and for being such a great friend. Former members of the lab, Changchun, Jyothsna, Karunakar and Mikael Nöjd, thank you for great company and discussions.

Present and former members of UCMM, I am grateful not only for the good suggestions and discussions during seminars, but also for sharing equipment and lunches with me. A special thank you to Manjushree, Christoph and Anne-Cecile, for great food, music and company. UCMM is not the same without you! P-A, your help with orders, deliveries and gossip has been priceless!

I am grateful to members of the surgery department and especially to Malin Sund, for her efforts during my midterm seminar and for bandaging my finger, and to Anna-Maria Lundgren, for all the kind help with everything from computer support to filling in forms. I am also very grateful I got to meet Hanna Nyström, Erik Lundberg, Oskar Franklin, Malin Jansson, and the super-friendly bunch of medical students that I shared office space with over the years.

Without the love and support from my family, none of this would have been possible. First and foremost Lotta, you are the love of my life and the best life companion I could ever wish for. Irma and Lars, älskade småskitar! Mom, Dad, Frida, Noa, Therese, Robert, Maggan, and all associated partners and kids, thank you for always being there for me. I love you all! Peter Blombäck, you were one of the most loving persons I ever knew. I hate it that you are not here to see the end of this. Rest in peace, dear father in law.

I am extremely grateful to all my dear friends outside work for all the dinner parties, baby-sittings and what not. What would I do without you? Britta Eland and Karin Anklew, thank you for getting me interested in science, so many years back.
REFERENCES


